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February 17, 2004

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Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Art Unit 1635

Re: U.S. Utility Patent Application
Application No. 09/275,883; Filed: March 25, 1999
For: **Inducible Alphaviral Gene Expression System**
Inventors: Renner *et al.*
Our Ref: 1700.0020001/JAG/FRC

Sir:

Transmitted herewith for appropriate action are the following documents:

1. Fee Transmittal Form (PTO/SB/17);

2. Brief on Appeal Under 37 C.F.R. § 1.192 (in triplicate) along with Exhibit 1;

3. PTO Credit Card Payment Form (PTO-2038) in the amount of **$330.00** to cover the brief on appeal fee; and

4. Return postcard.

It is respectfully requested that the attached postcard be stamped with the date of filing of these documents, and that it be returned to our courier. In the event that extensions of time are necessary to prevent abandonment of this patent application, then such extensions of time are hereby petitioned.

The U.S. Patent and Trademark Office is hereby authorized to charge any fee deficiency, or credit any overpayment, to our Deposit Account No. 19-0036.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Frank R. Cottingham

Frank R. Cottingham
Attorney for Applicants
Registration No. 50,437

FRC/pcd
Encls.

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PTO/SB/17 (10-03)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 330.00

Complete if Known	
Application Number	09/275,883
Filing Date	March 25, 1999
First Named Inventor	Renner et al.
Examiner Name	Schnizer, R.
Art Unit	1635
Attorney Docket No.	1700.0020001/JAG/FRC

OIPE FEB 17 2004 PATENT & TRADEMARK OFFICE

METHOD OF PAYMENT *(check all that apply)*

☐ Check ☒ Credit card ☐ Money Order ☒ Other** ☐ None

☐ Deposit Account: **Charge any deficiencies or credit any overpayments in the fees to Deposit Acct. No. 19-0036.*

Deposit Account Number: 19-0036

Deposit Account Name: Sterne Kessler, Goldstein & Fox P.L.L.C.

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	

SUBTOTAL (1) ($)

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

		Extra Claims	Fee from below	Fee Paid
Total Claims	- 20 **=		x	=
Independent Claims	- 3 **=		x	=
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($)

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	330.00
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) ____________

*Reduced by Basic Filing Fee Paid **SUBTOTAL (3)** ($) 330.00

SUBMITTED BY				(Complete *(if applicable)*)	
Name *(Print/Type)*	Frank R. Cottingham	Registration No. *(Attorney/Agent)*	50,437	Telephone	(202) 371-2600
Signature	Frank R. Cottingham			Date	FEB. 17, 2004

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

This collection of information is required by 37 CFR 1.17 and 1.27. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 1349
RENNER *et al.*	Art Unit: 1635
Appl. No. 09/275,883	Examiner: Schnizer, R.
Filed: March 25, 1999	Atty. Docket: 1700.0020001/JAG/FRC
For: **Inducible Alphaviral Gene Expression System**	

Brief on Appeal Under 37 C.F.R. § 1.192

Mail Stop Appeal Brief - Patents

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

A Notice of Appeal from the rejection of claims 75-78, 81-84, 86-101, 103, 105-107 and 109-145 (twice rejected) was filed on December 17, 2003. Appellants hereby file this Appeal Brief in triplicate, together with the required brief filing fee.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Table of Contents

I. Real Party in Interest (37 C.F.R. § 1.192(c)(1))

The real party in interest in this appeal is Cytos Biotechnology AG.

II. Related Appeals and Interferences (37 C.F.R. § 1.192(c)(2))

Appellants' undersigned representative is not aware of any appeals or interferences related to this application.

III. Status of Claims (37 C.F.R. § 1.192(c)(3))

The present application was filed on March 25, 1999. As originally filed, the application contained a total of 74 claims.

In an Amendment filed November 15, 2000, claims 1-74 were cancelled, and claims 75-125 were added.

In an Amendment filed July 31, 2001, claims 80, 85, 104 and 108 were cancelled, claims 75, 81, 103 and 125 were amended, and claims 126-136 were added.

In an Amendment filed November 4, 2002, claim 79 was cancelled, claims 75, 81, 82, 86, 93, 94, 97, 98, 100, 101, 103, 105, 109, 116, 117, 120, 121, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135 and 136 were amended, and claims 137-145 were added.

Claims 75-78, 81-84, 86-103, 105-107 and 109-145 are pending in the application. Claim 102 is allowed. Claims 75-78, 81-84, 86-101, 103, 105-107 and 109-145 are now on appeal. A copy of the claims on appeal can be found in the attached Appendix.

IV. Status of Amendments (37 C.F.R. § 1.192(c)(4))

All amendments have been entered. No amendments have been filed subsequent to the issuance of the Office Action dated June 17, 2003.

V. Summary of Invention (37 C.F.R. § 1.192(c)(5))

A. Background

Alphaviruses have a positive-sense RNA genome. The 5' two-thirds of the alphaviral genome encodes four nonstructural proteins, nsP1 through nsP4. The nonstructural proteins together make up the alphaviral replicase which replicates the alphaviral genome in the cytoplasm of infected host cells. The 3' one-third of the genome encodes the structural proteins which form the capsid and envelope protein that encloses the alphaviral genome.

Investigators have modified the alphaviral genome to allow high levels of transgene expression. The modified alphaviral genome, known as a replicon, is an RNA molecule comprising the replicase-encoding region (nsPs1-4) and at least one *cis*-acting sequence element to which the replicase binds to initiate RNA replication. The region encoding the structural proteins is replaced with a heterologous sequence or gene of interest.

Replicons are introduced into host cells. In the cytoplasm of the host cell, a complementary negative-strand RNA molecule is synthesized from the replicon by the alphaviral replicase. The negative strand in turn serves as the template for two distinct positive-strand RNA species. One species corresponds to the entire replicon. The second RNA species corresponds to the 3' region of the replicon encoding the heterologous sequence or gene of interest. The second RNA species is known as the

subgenomic RNA species. The gene product of interst is translated from the subgenomic RNA species.

Originally, alphaviral replicons (RNA molecules) were transcribed *in vitro* before being introduced into host cells. More recently, DNA-based alphaviral vectors (plasmids) have been developed. DNA-based alphaviral vectors are transfected into host cells. The replicon is transcribed from the DNA in the nucleus of the host cell and is subsequently transported into the cytoplasm. Replication and transgene expression from the replicon take place in the cytoplasm.

Alphaviral replicases, however, are lethal to cells. Gene expression from traditional alphaviral vectors (including RNA-based and DNA based vectors) is therefore only transient.

B. The Present Invention

The present invention avoids the cell lethality problems that are encountered with traditional alphaviral expression vectors. The present invention overcomes the cell lethality problems by taking advantage of mutations in the alphaviral genome which render the alphaviral replicase non-cytopathic and temperature sensitive. The alphaviral replicases encoded from the vectors of the present invention are not lethal to cells and only function at certain temperatures. These characteristics allow for much greater transgene expression than is possible with the alphaviral vectors previously known in the art.

More specifically, the present invention is directed to DNA molecules and vector systems which encode RNA molecules comprising: (a) at least one *cis*-acting sequence

element, (b) a first open reading frame which encodes a non-cytopathic temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of said replicase, and (c) at least one second nucleotide sequence selected from the group consisting of: (i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a translatable format after one or more RNA-dependent RNA replication events; (ii) a sequence complementary to all or part of the second open reading frame of (i); and (iii) a sequence encoding an untranslated RNA molecule, or complement thereof; wherein said second nucleotide sequence is operably linked to a promoter which is recognized by said non-cytopathic, temperature-sensitive alphaviral replicase (claims 75-78, 81-84, 103, 105-107 and 125-136). Support for these aspects of the invention can be found throughout the specification, for example, at page 7, lines 4-17, and at page 21, line 23, through page 22, line 25.

The invention also includes methods involving the use of the DNA molecules/vector systems of the invention. The invention includes methods of making a recombinant host cell (claims 86 and 109). Support for this aspect of the invention can be found throughout the specification, for example, at page 8, lines 14-15. The invention includes methods for producing a protein or an untranslated RNA molecule (claims 93-96, 98, 99, 116-119, 121 and 122). Support for this aspect of the invention can be found throughout the specification, for example, at page 8, lines 23-27. The invention includes methods for producing alphaviral particles (claims 97 and 120). Support for this aspect of the invention can be found throughout the specification, for example, at page 8, lines 3-7. The invention includes methods for regulating the expression of a protein or an

untranslated RNA molecule in a recombinant host cell (claims 100, 101, 123 and 124). Support for this aspect of the invention can be found throughout the specification, for example, at page 22, line 26, through page 23, line 21.

The invention also includes *in vitro* cell cultures comprising recombinant host cells comprising the DNA molecules of the invention (claims 87-89, 92, 110-112 and 115). Support for this aspect of the invention can be found throughout the specification, for example, at page 29, line 1, through page 30, line 13. The invention also includes RNA molecules transcribed from the DNA molecules of the invention, and compositions comprising such RNA molecules (claims 90 and 113), and alphaviral particles containing RNA molecules transcribed from the DNA molecules of the invention (claims 91 and 114). Support for these aspects of the invention can be found throughout the specification, for example, at page 8, lines 3-7.

The invention also includes the DNA molecules/vector systems of the invention, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of the replicase (claims 137, 140 and 143). The invention also includes the DNA molecules/vector systems of the invention, wherein temperature sensitivity is conferred by one or more mutations in the nsP4 gene of the replicase (claims 138, 141 and 144). The invention also includes the DNA molecules/vector systems of the invention, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of the replicase and temperature sensitivity is conferred by one or more mutations in the nsP4 gene of the replicase (claims 139, 142 and 145). Support for these aspects of the invention can be found throughout the specification, for example, at page 21, line 23, through page 22, line 25.

VI. Issues on Appeal (37 C.F.R. § 1.192(c)(6))

A. Written Description

The first issue on appeal is whether claims 75-78, 81-84, 86-101, 103, 105-107 and 109-145 contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

B. Enablement

The second issue on appeal is whether the specification would have enabled one of ordinary skill in the art to which the invention pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with claims 75-78, 81-84, 86-101, 103, 105-107 and 109-136.

VII. Grouping of Claims (37 C.F.R. § 1.192(c)(7))

For the purpose of this appeal, the claims do not stand or fall together. The claims are grouped as follows:

- Group I: Claims 75-78, 81-84, 86-101, 103, 105-107 and 109-136; and
- Group II: Claims 137-145.

VIII. Argument (37 C.F.R. § 1.192(c)(8))

A. Written Description

1. Legal Standard for Written Description

To satisfy the written description requirement of 35 USC § 112, first paragraph, an Applicant must convey with reasonable clarity to those skilled in the art that, as of the effective filing date, the Applicant was in possession of the invention. *See Vas-Cath,*

Inc. v. Mahurkar, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). As made clear by the Federal Circuit, "[t]he written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed.'" *Union Oil Co. of Cal. v. Atlantic Richfield Co.*, 208 F.3d 989, 997, 54 USPQ2d 1227, 1232 (Fed. Cir. 2000); *see also Amgen Inc. v. Hoechst Marion Roussel Inc.*, 65 USPQ2d 1385, 1397 (Fed. Cir. 2003) ("[t]he purpose of the written description requirement is to prevent an applicant from later asserting that he invented that which he did not.")

The Federal Circuit has recently adopted the standard for determining compliance with the written description requirement as set forth in the USPTO's "Guidelines for the Examination of Patent Applications under 35 U.S.C. § 112, first paragraph, Written Description Requirement." *See Enzo Biochem, Inc. v. Gen-Probe Inc.*, 296 F.3d 1316, 1324, 63 USPQ2d 1609, 1613 (Fed. Cir. 2002). According to the USPTO's Guidelines:

> An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.

MPEP § 2163; *See also, Enzo*, 296 F.3d at 1324, 63 USPQ2d at 1613.

2. ***The Written Description Requirement Is Fully Satisfied for the Subject Matter of the Claims of Group I (Claims 75-78, 81-84, 86-101, 103, 105-107 and 109-136)***

(a) ***The USPTO's Written Description Guidelines are Satisfied for the Claims of Group I***

(i) ***The Written Description Requirement May be Satisfied By Disclosure of Structural Characteristics, Functional Characteristics, and a Known or Disclosed Correlation Between Function and Structure***

The USPTO's Written Description Guidelines indicate that the written description requirement of 35 U.S.C. § 112, first paragraph, may be satisfied by the disclosure of relevant identifying structural characteristics and/or functional characteristics when there is a known or disclosed correlation between function and structure. *See* MPEP § 2163. As discussed in more detail below, the present specification discloses relevant identifying structural and functional characteristics of the claimed invention. In addition, the claims provide a structural and functional definition of the invention. Furthermore, there is a known correlation between the function of the nucleic acid molecules of the invention and their structure. Therefore, an analysis based on the USPTO's Written Description Guidelines compels the conclusion that the claimed invention is more than adequately described by the specification.

(ii) ***The Specification Discloses the Structural and Functional Characteristics of the Subject Matter Encompassed by the Claims***

The claims of Group I are directed to, or involve the use of, nucleic acid molecules which comprise a first open reading frame which encodes a non-cytopathic, temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the non-

structural proteins of the replicase. The present specification discloses the structural and functional characteristics of the claimed invention.

The specification provides a working example of a nucleic acid molecule that encodes a non-cytopathic, temperature sensitive alphaviral replicase. The construction of this nucleic acid molecule, designated pCYTts, is described in the specification at page 41, line 2, through page 43, line 8 (Example 1). This exemplary nucleic acid molecule encodes a Sindbis virus replicase containing a Pro726Ser mutation in nsP2 and a Gly153Glu mutation in nsP4. It was further demonstrated that pCYTts, containing various heterologous genes, is non-cytopathic and allows temperature sensitive transgene expression. *See* specification at page 43, line 10, through page 51, line 9 (Examples 2-6). The complete nucleotide sequence of pCYTts is set forth in the specification at Figures 3A-3D (SEQ ID NO:1).

Based on the working examples in the specification, a person of ordinary skill in the art would recognize the pertinent functional and structural characteristics of the invention. That is, a person of ordinary skill in the art would recognize that the nucleic acid molecules of the invention encode a temperature sensitive, non-cytopathic replicase, and that non-cytopathicity and temperature sensitivity are conferred by mutations in the nonstructural protein genes of the replicase.

In other places, the specification makes explicit the fact that the invention includes nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases, and that non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of

the replicase. For example, the specification provides the following summary of the invention:

> The present invention provides compositions and methods for regulated expression of proteins or untranslated RNA molecules in recombinant host cells. More specifically, the present invention provides polynucleotides and methods which allow precise regulation of the amount of specific RNA molecules produced in stably transfected recombinant host cells. This precise regulation results from the use of a temperature-sensitive RNA-dependent RNA polymerase (*i.e.*, a replicase) which only replicates RNA molecules, to form new RNA molecules, at permissive temperatures.

See specification at page 6, lines 17-24. It is further noted that:

> When using alphavirus replicase proteins, in most instances, it is desirable to convert the cytopathic phenotype of the replicase protein to a non-cytopathic phenotype. Preferred mutations which confer such a phenotype are in the nsp2 gene (*e.g.*, the proline residue at position 726 is replaced with a serine residue). Mutations are known in the art which render the replicase protein non-cytopathic (Weiss *et al.*, *J. Virol. 33*:463-474 (1980); Dryga *et al.*, *Virology 228*:74-83 (1997)). These mutations may be introduced by a number of means, including site directed mutagenesis.
>
> As noted above, when a non-cytopathic Sindbis virus replicase is used in the practice of the invention, a mutation may be introduced in the nsp2 gene. One such mutation results from the exchange of the proline residue at position 726 to another of the 20 natural occurring amino acids, such as a serine (abbreviated as "Pro 726 Ser"). *Alternatively, any other mutation rendering the replicase molecule non-cytopathic is within the scope of the invention.* The creation and the identification of mutations which render the Sindbis replicase non-cytopathic are described in more detail elsewhere (Weiss *et al., J. Virol. 33*:463-474 (1980); Dryga *et al., Virology 228*:74-83 (1997); patent application WO 97/38087). Further, methods for inducing such mutations are known in the art (*see, e.g.*, Sambrook, J. *et al.*, eds., MOLECULAR CLONING,

> A LABORATORY MANUAL, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997)).

See specification at page 21, line 23, through page 22, line 12 (emphasis added).

With respect to temperature sensitivity, it is noted that:

> Temperature sensitivity (*ts*) may be conferred, for example, by the introduction of a mutation in the nsp4 gene of the replicase. Preferably, mutations which confer a temperature-sensitive phenotype upon replicase activities are in a protein in complementation group F (Lemm *et al.*, *J. Virol.* *64*:3001-3011 (1990)). For example, a temperature-sensitive phenotype may be conferred by changing Gly 153 of nsp4 to Glu. *Additionally, any other mutation which renders replicase activity temperature-sensitive can be used in the practice of the invention.* Methods for creating and identifying new temperature-sensitive mutants are described by Pfefferkorn (Burge and Pfefferkorn, *Virol.* *30*:204-213(1966); Burge and Pfefferkorn, *Virol.* *30*:214-223 (1966)). Further, any method useful for producing and identifying *ts* mutants which allow for the temperature-sensitive regulation of replicase activity can be employed to generate and isolate such mutants.

See specification at page 22, lines 13-25 (emphasis added).

Thus, it is clear from the specification that the invention encompasses nucleic acid molecules that encode non-cytopathic, temperature-sensitive alphaviral replicases. It is also clear from the specification that the properties of non-cytopathicity and temperature sensitivity can be conferred upon the replicases by *any mutation(s)* that render the replicase non-cytopathic and temperature sensitive. Methods for obtaining

such mutations were well known to persons of ordinary skill in the art as of the effective filing date of the application. *See* discussion below.

(iii) There is a Known Correlation Between Function (Non-Cytopathic and Temperature Sensitivity) and Structure (One or More Mutations in the Genes Encoding the Nonstructural Proteins of the Replicase)

The correlation between (a) the phenotypes of non-cytopathicity and temperature sensitivity and (b) mutations in the genes encoding the nonstructural proteins of alphaviral replicases was known in the art as of the effective filing date of the present application. Thus, in the context of the present claims, there was a known correlation between function and structure.

There are numerous examples in the scientific literature of mutations in alphaviral nsP genes that cause temperature-sensitivity and/or non-cytopathicity. Examples of such mutations are described in Suopanki *et al.*, *J. Gen. Virol. 79*:309-319 (1998); LaStarza *et al.*, *J. Virol. 68*:5781-5791 (1994); Wang *et al.*, *J. Virol. 65*:985-988 (1991); Shirako and Strauss, *Virology 177*:54-64 (1990); Hardy *et al.*, *Virology 177*:199-208 (1990); Hahn *et al.*, *J. Virol. 63*:3142-3150 (1989); Sawicki and Sawicki, *J. Virol. 67*:3605-3610 (1993); Hahn *et al.*, *J. Virol. 63*:1194-1202 (1989); Shirako and Strauss, *J. Virol. 72*:2310-2315 (1998); Lemm *et al.*, *J. Virol. 64*:3001-3011 (1990); Sawicki *et al.*, *Virology 174*:43-52 (1990) (copies submitted with Appellants' Supplemental Reply filed on March 31, 2003, as Exhibits 1-11, respectively); and Dryga *et al.*, *Virology 228*:74-83 (1997) (copy submitted as document AS6 in the Information Disclosure Statement filed on June 28, 1999). Thus, it was recognized in the art that temperature sensitivity and non-cytopathicity were conferred by mutations in the nsP genes of alphaviral replicases.

Additional mutations that render an alphaviral replicase non-cytopathic and temperature sensitive could have easily been obtained by persons of ordinary skill in the art. The ability of those skilled in the art to obtain non-cytopathic, temperature-sensitive alphaviral replicases is described in the Declaration of Dr. Sondra Schlesinger Under 37 C.F.R. § 1.132 (copy submitted with Appellants' Supplemental Reply filed on March 31, 2003) and is also addressed in the response to the Enablement rejection below.

In view of the numerous examples of nucleic acid molecules encoding non-cytopathic and/or temperature sensitive alphaviral replicases having mutations in the non-structural protein genes, and the ease with which one of ordinary skill in the art could have obtained additional non-cytopathic, temperature sensitive alphaviral replicases (*see* section VIII.B.2, below), it must be concluded that there was a known correlation between the function and structure of non-cytopathic, temperature sensitive alphaviral replicases.

(b) The Sindbis Virus Mutations Disclosed in the Specification Would Have Directed Persons of Ordinary Skill in the Art to Additional Non-Cytopathic, Temperature Sensitive Mutations in Other Alphaviruses, Due to the High Degree of Sequence Homology Shared Among Alphaviral Nonstructural Proteins

The working example in the specification involves a Sindbis virus non-cytopathic, temperature-sensitive replicase. The nsPs from various alphaviruses share a high degree of sequence homology. The high level of sequence homology that exists among the nsPs of alphaviruses is illustrated in the table, submitted with Appellants' Supplemental Reply filed on March 31, 2003, as Exhibit 13. This table shows the percent amino acid sequence identity and similarity that exists between the nsPs of the following alphaviruses: O'nyong-nyong virus (ONG), Ross River virus (RRV), Semliki

Forest virus (SFV), Sindbis virus (SinV), Venezuelan equine encephalitis virus (VEEV) and Western equine encephalomyelitis virus (WEEV). Appellants have also submitted individual amino acid sequence alignments comparing the sequences of alphaviral nsP1, nsP2, nsP3 and nsP4 proteins. *See* Appellants' Supplemental Reply filed on March 31, 2003, Exhibits 14, 15, 16 and 17, respectively). Finally, a phylogenetic tree was presented showing the evolutionary relationship among the nsP2 and nsP4 proteins from various alphaviruses. *See* Appellants' Supplemental Reply filed on March 31, 2003, Exhibit 18. Although these sequence comparisons involve amino acid sequences, they necessarily reflect the high degree of correlation that exists among the alphaviral nsP genes at the nucleic acid level.

In view of the high degree of sequence homology that exists among the nsPs of alphaviruses, the exemplary mutations disclosed in the specification would have directed persons of ordinary skill in the art to the nucleotide positions in other alphaviruses that, when mutated, would likely result in non-cytopathicity and temperature sensitivity. Thus, although the specification describes a nucleic acid molecule that encodes a non-cytopathic, temperature sensitive *Sindbis virus* replicase, the exemplary disclosed species would have been regarded by persons of ordinary skill in the art as, in effect, a disclosure of non-cytopathic, temperature sensitive mutations in the nonstructural protein genes of *all* alphaviruses. In view of the exemplary embodiment provided in the specification, and the high degree of sequence homology that exists among alphaviral nsPs, a person of ordinary skill in the art would conclude that Appellants were in possession of the claimed subject matter.

(c) Summary

The written description requirement of 35 U.S.C. § 112, first paragraph is fully satisfied for the subject matter of the claims of Group I. The USPTO's guidelines indicate that the written description requirement may be satisfied by the disclosure of functional and structural characteristics when there is a known or disclosed correlation between function structure. Here, Appellants have disclosed both the functional characteristics (non-cytopathicity and temperature sensitivity) and the structural characteristics (one or more mutations in the genes encoding the nonstructural proteins of the replicase) of the claimed subject matter. It was well known in the art that mutations in alphaviral nonstructural protein genes cause non-cytopathicity and/or temperature sensitivity. Thus, there is a known correlation between function and structure. In addition, the disclosure of an exemplary nucleic acid molecule that encodes a non-cytopathic, temperature sensitive Sindbis virus replicase (pCYTts) would have been regarded by persons of ordinary skill in the art as effectively disclosing non-cytopathic, temperature sensitive mutations in all alphavirus species because of the high level of sequence homology that exists among the nonstructural proteins of alphaviruses. Thus, the written description requirement of 35 U.S.C. § 112, first paragraph, is fully satisfied for the subject matter of the claims of Group I.

3. The Written Description Requirement Is Fully Satisfied for the Subject Matter of the Claims of Group II (Claims 137-145)

The claims of Group II depend from Group I claims 75, 103 and 125. The subject matter of the claims of Group II is adequately described in the specification for at least the same reasons that the subject matter of the claims of Group I is adequately described. *See* discussion in section VIII.A.2, above. In addition, the claims of Group

II specify that non-cytopathicity is conferred by one or more mutations in the nsP2 gene of the replicase, and/or that temperature sensitivity is conferred by one or more mutations in the nsP4 gene of the replicase. Thus, the claims of Group II provide even more structural definition for the nucleic acid molecules encoding the alphaviral replicase than do the claims of Group I. The correlation between structure and function is therefore even more evident for the claims of Group II. In view of the high level of structural definition provided for the nucleic acid molecules in the claims of Group II, a person of ordinary skill in the art would appreciate that Appellants were in possession of the subject matter of these claims. The written description requirement of 35 U.S.C. § 112, first paragraph, is fully satisfied for the claims of Group II.

4. The Examiner Has Not Established a Prima Facie Case of Insufficient Written Description

(a) Satisfaction of the Written Description Requirement Does Not Necessarily Require the Disclosure of Multiple Working Examples

The Examiner has not established a *prima facie* case of insufficient written description. The Examiner has based the written description rejection on the absence of multiple working examples of non-cytopathic, temperature-sensitive alphaviral replicases in the specification. *See* Office Action dated June 17, 2003, page 4, lines 1-4. According to the Examiner, "[t]he central issue in this analysis is whether Applicant has disclosed a number of species which is representative of the claimed genus." *See* Office Action dated June 17, 2003, page 4, lines 1-2. The Examiner has cited the Written Description Guidelines to support this assertion. *See* Office Action dated June 17, 2003, page 3, lines 12-14.

Satisfaction of the written description requirement, however, does not necessarily require the disclosure of multiple working examples. There are other factors that must be taken into consideration such as, *e.g.*, the disclosure of structural and functional characteristics and the correlation between structure and function. *See* MPEP § 2163; *see also Enzo*, 296 F.3d at 1324, 63 USPQ2d at 1613. As noted above, the present specification discloses (a) the functional characteristics of the nucleic acid molecules included in the invention (*i.e.*, that they encode a non-cytopathic, temperature sensitive alphaviral replicase); and (b) the structural characteristics of the nucleic acid molecules included in the invention (*i.e.*, that non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of the replicase). In addition, it was well known in the art that non-cytopathicity and temperature sensitivity are caused by mutations in the genes encoding the nonstructural proteins of alphaviral replicases. *See* section VIII.A.2(a)(iii), above. Thus, there was a known correlation between the function of the alphaviral replicases of the invention and their structure. The absence of multiple working examples cannot, by itself, support a *prima facie* case of insufficient written description.

(b) Satisfaction of the Written Description Requirement Does Not Require the Disclosure of All Mutations that Render Alphaviral Replicases Non-Cytopathic and Temperature Sensitive

The Examiner also stated that "the specification has failed to disclose what mutations are required to render any other RNA-dependent RNA polymerase both temperature sensitive and non-cytopathic, or what other mutations could confer this phenotype on the Sindbis virus polymerase." *See* Office Action dated June 17, 2003, page 4, lines 9-12. As mentioned above, it was known that mutations in the

nonstructural protein genes of alphaviruses produce non-cytopathicity and/or temperature sensitivity, and that a person of ordinary skill in the art, in view of the present specification, could have easily obtained nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases. Satisfaction of the written description requirement therefore does not require that all possible mutations that confer these phenotypes be recited in the specification.

The Examiner stated that:

> [t]he instant application does not provide a written description that would allow one of skill in the art to immediately *envisage* the specific structure for Sindbis virus non-cytopathic, temperature sensitive replicase, or for the broader genus of alphaviral non-cytopathic, temperature-sensitive replicase.

Office Action dated June 17, 2003, page 5, lines 15-18 (emphasis added). Along these same lines, the Examiner also asserted that:

> The disclosed mutations do not provide a sufficient correlation between structure and function to allow one to *envisage* other temperature sensitive mutations, and therefore they do not provide an adequate written description of the genus of temperature sensitive alphaviral replicases.

Office Action dated June 17, 2003, page 8, lines 4-7 (emphasis added). Appellants respectfully submit that the proper legal standard for written description does not require that one of ordinary skill in the art be able to "envisage," *i.e.*, "to picture in the mind" (American Heritage Dictionary), the nucleic acid sequence of every nucleic acid molecule that is encompassed by or included within the present claims. The Examiner has failed to point to any legal authority to support such a restrictive view of the written

description requirement when the claims include a structural as well as a functional definition of the invention. Cases such as *Amgen* and *Fiers v. Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993), which were cited by the Examiner, involve claims/counts that defined the invention by *functional* language only. These cases therefore do not support a rejection of the present claims for insufficient written description.

In fact, the USPTO's own guidelines clearly indicate that functional characteristics *alone* may satisfy the written description requirement if there is a known or disclosed correlation between structure and function. *See* MPEP § 2163. This position has been explicitly endorsed by the Federal Circuit in the context of inventions involving genetic material. *See Enzo*, 296 F.3d at 1324, 63 USPQ2d at 1613.

Here, Appellants have provided, not only a functional description of the nucleic acid molecules of the invention (encoding a non-cytopathic, temperature sensitive alphaviral replicase), but also structural characteristics of the nucleic acid molecules (non-cytopathicity and temperature sensitivity being conferred by one or more mutations in the genes encoding the nonstructural proteins of the replicase). The claims of Group II provide even more structural definition, specifying that non-cytopathicity is conferred by one or more mutations in the nsP2 gene of the replicase, and/or that temperature sensitivity is conferred by one or more mutations in the nsP4 gene of the replicase. As demonstrated above, it was well known in the art that non-cytopathicity and temperature sensitivity are caused by mutations in the genes encoding the nonstructural proteins of alphaviral replicases. *See* section VIII.A.2(a)(iii), above. Thus, there is a known correlation between (a) non-cytopathicity and temperature sensitivity of alphaviral

replicases on the one hand, and (b) mutations in the genes encoding the nonstructural proteins of the replicase on the other.

Thus, when analyzed under the appropriate legal standard as specified by the Federal Circuit, and the USPTO's guidelines, it must be concluded that the written description requirement is fully satisfied for the claims on appeal. The Examiner's requirement for a detailed structural definition of every member of a claimed genus is not the proper legal standard and cannot stand as the basis for a rejection under 35 U.S.C. § 112, first paragraph.

B. Enablement

1. Legal Standard for Enablement

In order to satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph, Appellants' specification must enable any person skilled in the art to make and use the claimed invention without undue experimentation. *See In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). *See also United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988). The factors to be considered when determining whether the necessary experimentation is "undue" include: (a) the breadth of the claims, (b) the nature of the invention, (c) the state of the prior art, (d) the level of one of ordinary skill, (e) the level of predictability in the art, (f) the amount of direction provided by the inventor, (g) the existence of working examples, and (h) the quantity of experimentation needed to make or use the invention based on the content of the disclosure. *See Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. Moreover, as long as the specification discloses at least one method for making and using the claimed invention, then the enablement requirement of 35 U.S.C. § 112, first

paragraph is satisfied. *See Johns Hopkins Univ. v. CellPro, Inc.*, 152 F.3d 1342, 1361, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998).

An Applicant is not limited to the confines of the specification to provide the necessary information to enable an invention. *See In re Howarth*, 654 F.2d 103, 105-6, 210 USPQ 689, 692 (CCPA 1981). An Applicant need not supply information that is well known in the art. *See Genentech, Inc. v. Novo Nordisk*, 108 F.3d 1361, 1366, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997); *Howarth*, 654 F.2d at 105-6, 210 USPQ at 692; *see also In re Brebner*, 455 F.2d 1402, 173 USPQ 169 (CCPA 1972) (finding a disclosure enabling because the procedure for making the starting material, although not disclosed, would have been known to one of ordinary skill in the art as evidenced by a Canadian patent). "'That which is common and well known is as if it were written out in the patent and delineated in the drawings.'" *Howarth*, 654 F.2d at 106, 210 USPQ at 692 (quoting *Webster Loom Co. v. Higgins* et al., 105 U.S. (15 Otto.) 580, 586 (1881)). Moreover, one of ordinary skill in the art is deemed to know not only what is considered well known in the art but also where to search for any needed starting materials. *See Id.*

In order to establish a *prima facie* case of lack of enablement, the Examiner has the initial burden to set forth a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). To satisfy this burden, "it is incumbent upon the Patent Office. . . to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See In re Marzocchi*, 439 F.2d 220, 224, 169

USPQ 367, 370 (CCPA 1971) (emphasis in original). As enunciated by the Federal Circuit:

> [A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

In re Brana, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (emphasis in original; quoting *Marzocchi*, 439 F.2d at 224, 169 USPQ at 370).

2. The Subject Matter of the Claims of Group I (Claims 75-78, 81-84, 86-101, 103, 105-107 and 109-136) is Fully Enabled

A person of ordinary skill in the art would have been able to make and use the full scope of the subject matter encompassed by the claims of Group I using only routine methods. More specifically, it would have required only routine experimentation to obtain nucleic acid molecules that encode, *inter alia*, a non-cytopathic, temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of the replicase. Thus, the enablement requirement of 35 U.S.C. § 112, first paragraph, is fully satisfied for the claims of Group I.

(a) Based on Examples in the Specification and The High Level of Sequence Homology Shared Among Alphaviral Nonstructural Proteins, A Person of Ordinary Skill in the Art Would Have Been Able to Make and Use the Full Scope of Subject Matter Encompassed by the Claims of Group I

The present specification describes in detail the construction of an exemplary nucleic acid molecule that encodes a non-cytopathic, temperature sensitive alphaviral replicase (pCYTts). *See* specification at page 41, line 2, through page 43, line 8 (Example 1). This exemplary nucleic acid molecule encodes a Sindbis virus replicase containing a Pro726Ser mutations in nsP2 and a Gly153Glu mutation in nsP4. In view of this working example, a person of ordinary skill in the art could have constructed, with only routine methods, additional nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases. For example, a skilled artisan, in view of the high level of sequence homology shared among nsPs from various alphaviruses (*see* section VIII.A.2(b), above), could have easily made mutations in the nsP genes of other alphaviruses which correspond to the nsP mutations described in the specification. The mutants could have been easily tested to confirm that they encoded non-cytopathic, temperature sensitive replicases. Such testing would not have been regarded as undue experimentation.

(b) A Person of Ordinary Skill in the Art Would Have Been Able to Make and Use the Full Scope of Subject Matter Encompassed by the Claims of Group I by Genetic Screening

(i) Genetic Screening Approaches for Producing Alphaviral Mutants with Particular Phenotypes Were Well Known in the Art as of the Effective Filing Date of the Application

In addition to using the working examples from the specification as a guide for creating nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases, a person of ordinary skill in the art could also have used genetic screening approaches.

Genetic screening approaches in general, and genetic screening approaches to produce temperature sensitive or non-cytopathic alphaviral mutants in particular, were well known to persons of ordinary skill in the art many years prior to the effective filing date of the present application. General methods for producing mutant proteins having particular desired phenotypes are described in Sambrook *et al.*, "Creating Many Mutations in a Defined Segment of DNA," in *Molecular Cloning, A Laboratory Manual*, Sambrook *et al.*, eds., Cold Spring Harbor Laboratory Press, pp. 15.95-15.108 (1989) (copy submitted with Appellants' Amendment and Reply Under 37 C.F.R. § 1.116, filed November 4, 2002, as Exhibit 1). Sambrook describes oligonucleotide-, chemical-, and polymerase-based mutagenesis methods. The methods of Sambrook could have been used to produce non-cytopathic, temperature sensitive alphaviral replicase mutants for use with the present invention.

In addition, a review of the scientific literature demonstrates that random mutagenesis and genetic screening approaches have been used for several years to

identify alphaviral mutants having specific desired phenotypes. *See, e.g.*, Keränen and Kääriäinen, *Acta Path. Microbiol. Scand. Sect. B, 82*:810-820 (1974) (copy submitted with the Reply Under 37 C.F.R. § 1.111, filed December 17, 2003, as Exhibit C). Keränen and Kääriäinen describe the identification of sixteen temperature sensitive Semliki Forest virus mutants. The mutants were identified by exposing virus particles to a mutagen and selecting for mutants that were defective in virus RNA synthesis at non-permissive temperatures.

Similarly, Weiss *et al., J. Virol. 33*:463-474 (1980) (copy submitted as document AR26 in the Information Disclosure Statement filed on June 28, 1999) used a genetic selection approach to identify Sindbis virus mutants capable of establishing persistent infection of BHK host cells (*i.e.*, non-cytopathic mutants). Subsequently, Dryga *et al.*, *Virology 228*:74-83 (1997) (copy submitted as document AS6 in the Information Disclosure Statement filed on June 28, 1999) identified the precise genetic change responsible for the non-cytopathic phenotype of the virus mutants identified by Weiss.

At the time of the effective filing date of the present application, a person of ordinary skill in the art, in view of the teachings of the present specification, could have combined the mutagenesis and selection approach used by Keränen and Kääriäinen (identifying temperature sensitive mutants) with that of Weiss (identifying non-cytopathic mutants) and Dryga to produce alphaviral replicases possessing both non-cytopathic and temperature sensitive phenotypes.

The Declaration of Dr. Schlesinger Under 37 C.F.R. § 1.132 (submitted with Appellants' Supplemental Reply, filed on March 31, 2003) provides additional support for Appellants' positions that (a) a person of ordinary skill in the art would have likely

used a mutagenesis and screening approach to obtain nucleic acid molecules encoding non-cytopathic, temperature-sensitive alphaviral replicases for use in the present invention, and (b) obtaining such nucleic acid molecules using a mutagenesis and screening approach would not have required undue experimentation.

As noted by the Federal Circuit, screening -- even screening that involves the generation of numerous negative outcomes -- is not deemed undue experimentation when those skilled in the art typically engage in such screening. *See Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. The references cited above and the Declaration of Professor Schlesinger show that persons of ordinary skill in the art typically engaged in genetic screening to identify alphavirus replicase mutants having desired phenotypes; *i.e.*, non-cytopathicity and temperature sensitivity. Under *Wands*, the production of nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases by genetic screening would not be regarded as involving undue experimentation.

(ii) Several Temperature Sensitive Alphaviral Replicases Were Known in the Art as of the Effective Filing Date of the Application

There are many examples in the art, prior to the effective filing date of the present application, of temperature sensitive alphaviral replicases that were created by mutagenesis and screening strategies. Examples include LaStarza *et al.*, *J. Virol. 68*:5781-5791 (1994) (describing the construction of random linker insertion mutations in Sindbis virus nsP3 to generate temperature sensitive mutants); Shirako and Strauss, *Virology 177*:54-64 (1990) (describing mutations created in the penultimate Gly in Sindbis virus nsP1 which resulted in temperature sensitivity); and Shirako and Strauss, *J. Virol. 72*:2310-2315 (1998) (describing N terminal mutations made in the nsP4 gene of

Sindbis virus which caused temperature sensitivity). The strategies used in these references would have been available to persons of ordinary skill in the art.

Moreover, in view of the guidance provided in the present specification, the temperature sensitive mutants that were known in the art (such as those described above) could have been combined with non-cytopathic mutations (*e.g.*, the Pro726Ser mutation disclosed in the specification, or other non-cytopathic mutations obtained through genetic screening approaches). The resulting combination of mutations would have resulted in nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases that could be used with the present invention. The ability of persons of ordinary skill in the art to combine temperature sensitive mutations with non-cytopathic mutations to produce mutant replicases having both phenotypes is described in section VIII.B.2(c)(ii), below.

(c) Expression Vectors Encoding Non-Cytopathic, Temperature Sensitive Alphaviral Replicases Have been Described In the Art After the Effective Filing Date of the Application, and Were Made Using Methods That Were Known and Available Prior to the Effective Filing Date of the Application

Publications dated after the effective filing date of an application can be used to support enablement when the later publications provide evidence of the state of the art existing on the filing date of the application. *See In re Hogan*, 559 F.2d 595, 605, 194 USPQ 527, 537 (CCPA 1977). As noted above, several non-cytopathic or temperature sensitive alphaviral replicase mutants were known in the art prior to the effective filing date of the application. Additional alphaviral expression vectors encoding non-cytopathic, temperature sensitive replicases have been described in publications dated after the effective filing date of the application. *See* section VIII.B.2(c)(ii), below. The

techniques, materials and information used to create the alphaviral expression vectors described in these post-filing date references, however, would have been available to persons of ordinary skill in the art *prior* to the effective filing date. Therefore, the examples in the art of the successful production of non-cytopathic, temperature sensitive alphaviral replicases demonstrate that making and using the nucleic acid molecules of the present invention would not have required undue experimentation.

(i) Several Non-Cytopathic Alphaviral Replicase Mutants Have Been Made Using Methods That Were Available to Persons of Ordinary Skill in the Art as of the Effective Filing Date of the Present Application

1) Weiss/Dryga

As discussed above, Weiss genetically selected for Sindbis viruses that persistently infected BHK cells. Briefly, Weiss infected BHK cells with a preparation of Sindbis virus containing "defective interfering" (DI) particles. Four days after infection, most of the infected cells had died. However, a small percentage of viable infected cells were recovered. From the viable cells, a non-cytopathic virus was isolated, designated SIN-1. In a subsequent publication, Dryga described the cDNA cloning and sequencing of the nonstructural protein genes of the SIN-1 virus. Dryga thereby identified the mutation in the Sinbis genome responsible for non-cytopathicity.

Both Weiss and Dryga were published prior to the effective filing date of the present application (March 27, 1998). Weiss was published in 1980, and Dryga was published in 1997. Therefore, the methods used by Weiss and Dryga would have been available to a person of ordinary skill in the art as of the effective filing date of the application. Using the teachings of the present specification, a person of ordinary skill in

the art could have used the screening and cloning methods described by Weiss and Dryga to obtain additional non-cytopathic alphavirus replicase mutations.

2) Agapov

An alternative method for identifying non-cytopathic alphaviral replicase mutants is described in Agapov *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 95*:12989-12994 (October, 1998) (cited by the Examiner on Form PTO-892 in the Office Action Dated August 15, 2000). Agapov transduced cells with Sindbis replicons containing the puromycin *N*-acetyltransferase (*PAC*) gene as a dominant selectable marker. Replicons were selected that produced populations of cells that were not cytopathic to cells (*i.e.*, replicons that produced pur^R cells). Using this selection method, a mutation was identified in the nsP2 gene in which Pro726 was changed to a Leu.

The methods used by Agapov would have been available to persons of ordinary skill in the art prior to the effective filing date of the present application. Methods for producing alphaviral replicons expressing selectable markers were known in the art. *See, e.g.*, Xiong *et al.*, *Science 243*:1188-1191 (1989) (describing Sindbis virus vectors engineered to express the chloramphenicol acetyltransferase (CAT) selectable marker gene) (copy submitted as document AS27 in the Information Disclosure Statement filed on June 28, 1999). Transfecting cells with a replicon expressing a selectable marker and selecting for mutants that are non-lethal to cells would have therefore been routine in the art. It is worth noting that Agapov was published only seven months after the effective filing date of the present application (March 27, 1998). The fact that Agapov was published soon after the effective filing date of the present application provides further

indication that the methods of Agapov were known and available prior to the effective filing date of the application.

Persons of ordinary skill in the art, in view of the teachings of the present specification, could have used the genetic selection approach of Agapov to obtain additional non-cytopathic alphaviral replicase mutations.

3) Perri

Perri *et al., J. Virol. 74*:9802-9807 (2000) (copy submitted with Appellants' Supplemental Reply filed on March 31, 2003, as Exhibit 12) describes another method for obtaining non-cytopathic alphaviral replicases using methods that would have been available to persons of ordinary skill in the art prior to the effective filing date of the present application. Perri used a genetic screening approach that resulted in the identification of five non-cytopathic alphaviral replicase mutants. To identify the mutants, Perri placed the neomycin phospotransferase gene (*neo*) under the control of the subgenomic promoter in both Sindbis virus- and Semliki Forest virus-derived replicons. Some of the DNA templates were subjected to random mutagenesis. Replicons were transcribed from the DNA templates, and the RNA replicons were transfected into BHK cells. The transfected cells were subjected to G418 selection; persistent, drug resistant colonies were selected in order to identify non-cytopathic replicons.

Using this method, Perri identified five non-cytopathic mutations, two in the Sindbis virus nsP2 gene, and three in the Semliki Forest virus nsP2 gene. For Sindbis virus replicons, the mutations were A1E ("S1") and P726T ("S2"). For Semliki Forest virus replicons, the mutations were L10T ("SF2A"), a deletion of D469 ("SF1B") and

L713P ("SF2C"). *See* Perri at 9804, Figure 2C. Thus, using a genetic screening approach, Perri was able to easily identify five non-cytopathic alphaviral replicases.

The methods used by Perri would have been available to persons of ordinary skill in the art prior to the effective filing date of the present application. As mentioned above, methods for producing alphaviral replicons expressing selectable markers were known in the art. Procedures for mutagenizing nucleic acid molecules were also known in the art. *See, e.g.*, Sambrook (discussed in section VIII.B.2(b)(i), above). Transfecting host cells with the mutagenized replicons and selecting for drug-resistant colonies would have involved only routine methods. Therefore, the methods of Perri could have been used by persons of ordinary skill in the art, prior to the effective filing date of the present application, to obtain additional non-cytopathic alphaviral replicase mutations.

(ii) Non-Cytopathic, Temperature Sensitive Alphaviral Replicase Mutants Have Been Created by Combining Known Replicase Mutants, Using Methods That Were Available to Persons of Ordinary Skill in the Art as of the Effective Filing Date of the Present Application

There are two examples from the scientific literature of the production of alphaviral vectors that express non-cytopathic, temperature sensitive alphaviral replicases. In both examples, the nucleic acid molecules encoding the non-cytopathic, temperature sensitive alphaviral replicases were produced by combining known alphaviral replicase mutations. Standard molecular biological techniques (available in the art prior to the effective filing date of the present application) were used to create the combination mutants. Thus, these two examples demonstrate that, with the guidance of the present specification, the production of nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases could have been accomplished

prior to the effective filing date of the present application without undue experimentation.

The first example is Lundstrom *et al.*, *Histochem. Cell. Biol. 115*:83-91 (2001) ("Lundstrom 2001") (copy submitted with Appellants' Supplemental Reply filed on December 17, 2003, as Exhibit A). Lundstrom 2001 describes the combination of a known non-cytopathic mutation, referred to as SFV(PD) (which has two point mutations in the nsP2 gene), with previously known temperature sensitive mutations. As expected, the combination mutants exhibited both non-cytopathic and temperature sensitive phenotypes.

The second example is Lundstrom *et al.*, *Mol. Ther. 7*:202-209 (2003) ("Lundstrom 2003") (copy submitted with Appellants' Supplemental Reply filed on December 17, 2003, as Exhibit B). Lundstrom 2003 describes the combination of the SFV(PD) mutation with Perri's L713P nsP2 non-cytopathic mutation. The resulting combination mutant, referred to as SFV(PD713P), exhibited both non-cytopathic and temperature sensitive phenotypes.

Both Lundstrom 2001 and Lundstrom 2003 used PCR-based site-directed mutagenesis techniques to produce the combination mutants. PCR-based site directed mutagenesis techniques were well known in the art prior to the effective filing date of the present application. *See, e.g.*, Stappert, J., "Methods for Generating Multiple Site-Directed Mutations *In Vitro*," in *PCR Technology: Current Innovations*, Griffin and Griffin, eds., CRC Press, pp. 59-67 (1994) (copy submitted herewith as Exhibit 1). The methods described in the Lundstrom references would have been available to persons of ordinary skill in the art, prior to the effective filing date of the present application, and

would have enabled the production of nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases. The Lundstrom references demonstrate that alphaviral replicase mutants exhibiting both temperature sensitivity and non-cytopathicity could have been easily made by combining previously known mutations in nsP genes.

(d) Summary

The above discussion illustrates the following points in support of the enablement of the present invention:

1. Several temperature sensitive alphaviral replicase mutations were known in the art prior to the effective filing date of the present invention;
2. Several non-cytopathic alphaviral replicase mutant have been described in the art. The methods used to obtain the non-cytopathic replicase mutations would have been available to persons of ordinary skill in the art prior to the effective filing date of the present invention; and
3. Alphaviral expression vectors encoding non-cytopathic, temperature sensitive alphaviral replicases have been obtained by combining, *e.g.*, known temperature sensitive mutations with known non-cytopathic mutations. The methods used to combine the known mutations would have been available to persons of ordinary skill in the art prior to the effective filing date of the present invention.

Therefore, at the time of the effective filing date of the present invention, a person of ordinary skill in the art could have produced alphaviral expression vectors encoding non-cytopathic, temperature sensitive alphaviral replicases using routine

methods. Such methods would not have involved undue experimentation. Accordingly, the subject matter of the claims of Group I is fully enabled.

3. *The Subject Matter of the Claims of Group II (Claims 137-145) is Fully Enabled*

The claims of Group II depend from Group I claims 75, 103 and 125. The subject matter of the claims of Group II is fully enabled for at least the same reasons that the subject matter of the claims of Group I is enabled. *See* discussion in section VIII.B.2, above. In addition, the claims of Group II specify that non-cytopathicity is conferred by one or more mutations in the nsP2 gene of the replicase, and/or that temperature sensitivity is conferred by one or more mutations in the nsP4 gene of the replicase. Thus, in order to obtain nucleic acid molecules for use with the invention defined by the claims of Group II, a person of ordinary skill in the art would have been directed to the specific genes of the alphaviral replicase in which to concentrate his or her mutagenesis efforts. The subject matter of the claims of Group II is therefore fully enabled. (Appellants note that the claims of Group II were not rejected for lack of enablement.)

4. *The Examiner Has Not Established a Prima Facie Case of Non-Enablement*

(a) *A Person of Ordinary Skill in the Art Would Not have Been Required to Predict the Effects of Mutations on Protein Function in Order to Make Nucleic Acid Molecules that Encode Non-Cytopathic, Temperature Sensitive Alphaviral Replicases*

The Examiner has not established a *prima facie* case of non-enablement. The Enablement rejection is based on the Examiner's assertion that it is difficult to predict the relationship between nucleic acid mutations and protein function. *See* Office Action

dated June 17, 2003, page 11, lines 14-19. Apparently, the Examiner believes that, in order to produce additional nucleic acid molecules encoding non-cytopathic, temperature-sensitive alphaviral replicases, a skilled artisan would have needed to make individual site-directed mutations in the replicase genes, and would have needed to know *a priori* that the mutations caused the desired phenotypes. Appellants respectfully disagree.

As discussed in section VIII.B.2(c), above, alphaviral vectors encoding non-cytopathic, temperature sensitive replicases -- in addition to the exemplary embodiment described in the present specification -- have been described in the art. The methods that were used to produce these vectors would have been known and available to persons of ordinary skill in the art prior to the effective filing date of the present application. The methods that were used to produce these vectors did not involve predicting the effects of particular mutations on protein function. Rather, as with the exemplary embodiment in the present specification, the non-cytopathic, temperature sensitive vectors that were described in the art after the effective filing date of the application were produced by combining known mutations that individually caused non-cytopathicity and/or temperature sensitivity. The individual non-cytopathic and temperature sensitive mutations were produced using classical genetic screening approaches that were known and available prior to the effective filing date of the present application. The mutations were combined using well known molecular biological techniques that were also known and available prior to the effective filing date of the present application.

Making and using the non-cytopathic, temperature sensitive alphaviral vector exemplified in the present application (pCYTts) and the vectors described by others after

the effective filing date of the present application did not require the ability to predict protein function from protein structure. Thus, the Examiner's comments regarding the ability of persons of ordinary skill to predict "which amino acid substitutions will confer temperature sensitivity and non-cytopathicity on a given polymerase" cannot support a *prima facie* case of non-enablement.

(b) The Results Obtained By Combining Mutations in a Yeast F1-ATPase Cannot be Used to Draw Conclusions About the Predictability of Combining Mutations in Alphaviral Replicases

To further support the enablement rejection, the Examiner has cited the results that were observed when multiple mutations in the yeast F1-ATPase beta subunit were combined with one another. *See* Office Action dated June 17, 2003, pages 11-12. According to the Examiner, combining mutations in the yeast F1-ATPase beta subunit "produced totally unpredictable results." Appellants respectfully submit that the results observed by combining mutations in a yeast F1-ATPase do not provide any indication as to the effects of combining mutations in alphaviral replicases. Although the experiments cited by the Examiner, relating to a yeast F1-ATPase, produced unpredictable results, the examples described in the present application, and the examples provided by Lundstrom 2001 and Lundstrom 2003 produced entirely predictable results. That is, the combination of alphaviral replicase mutations produced alphaviral replicases that had both temperature sensitive and non-cytopathic phenotypes.

In summary, the present claims relate to alphaviral expression vectors encoding non-cytopathic, temperature sensitive alphaviral replicases. The combination of non-cytopathic mutations with temperature sensitive mutations, as described by the present inventors and by others, resulted in nucleic acid molecules encoding non-cytopathic,

temperature sensitive alphaviral replicases, as expected. The present claims do not relate to yeast F1-ATPases. Thus the results cited by the Examiner for a yeast F1-ATPase cannot support a *prima facie* case of non-enablement.

(c) The Sindbis Virus Replicase Mutations Described in the Specification Would Have Enabled the Production of Non-Cytopathic, Temperature Sensitive Replicases in Other Alphaviruses Due to the High Degree of Sequence Homology that is Shared Among Nonstructural Proteins of Alphaviruses

To Support the enablement rejection, the Examiner further stated that:

> Applicant has disclosed mutations only of a Sindbis virus replicase, whereas the claims encompass replicases from all alphaviruses. One of skill in the art could not predict which, if any, of these replicases could be mutated to be appropriately temperature sensitive and non-cytopathic, or what mutations would be required for this.

Office Action dated June 17, page 12, line 21, through page 13, line 2. Appellants again note that a person of ordinary skill in the art could have obtained nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases using genetic screening approaches. Such approaches could have been applied to any alphaviral replicase, regardless of the virus from which it was derived. In addition, alphavirus replicases share a great deal of sequence homology with one another. *See* Exhibits 13-18, submitted with Appellants' Supplemental Reply filed on March 31, 2003. Mutations in one alphaviral species which cause non-cytopathicity and/or temperature sensitivity would guide the skilled artisan to similar mutations at homologous genetic loci in other species to produce these phenotypes. Thus, the fact that the claims are not limited to non-cytopathic, temperature sensitive replicases from any particular alphavirus does not support a finding of non-enablement.

(d) Several Mutations Have been Identified that Render Alphaviral Replicases Non-Cytopathic, And Such Mutations Have Been Combined with Other Replicase Mutations to Produce Non-Cytopathic, Temperature Sensitive Alphaviral Replicases

Finally, the Examiner stated that "only a single alphaviral replicase mutation conferring non-cytopathicity has been identified, nsp2 P726S." *See* Office Action dated June 17, 2003, page 11, lines 5-6. Applicants respectfully point out that this is an incorrect statement. As discussed above, multiple non-cytopathic alphaviral replicase mutants have been identified by Weiss, Dryga, Agapov and Perri. *See* section VIII.B.2(c)(i), above. Non-cytopathic mutations of Perri and Lunstrom 1999 have been combined with other replicase mutations to produce nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases. *See* section VIII.B.2(c)(ii), above. Thus, the enablement rejection, insofar as it depends on the Examiner's position that only one non-cytopathic alphaviral replicase mutant has been identified, cannot be maintained.

(e) Summary

The Examiner has not presented sufficient evidence or scientific reasoning to demonstrate that it would have required undue experimentation to produce additional nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases. The fact that others in the art were able to produce additional non-cytopathic, temperature sensitive alphaviral replicases, using general methods that would have been available prior to the effective filing date of the present application, indicates that the production of such replicases would not have been regarded as involving undue experimentation. Thus, a *prima facie* case of non-enablement has not been established.

IX. Conclusion

In view of the foregoing remarks, Appellants respectfully request that the Board reverse the Examiner's 35 U.S.C. 112, first paragraph, rejections of claims 75-78, 81-84, 86-101, 103, 105-107 and 109-145 and remand this application for issue.

Respectfully submitted,
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X. Appendix (37 C.F.R. § 1.192(c)(9))

75. A DNA molecule which encodes an RNA molecule comprising:

(a) at least one *cis*-acting sequence element,

(b) a first open reading frame which encodes a non-cytopathic temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of said replicase, and

(c) at least one second nucleotide sequence selected from the group consisting of:

(i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a translatable format after one or more RNA-dependent RNA replication events;

(ii) a sequence complementary to all or part of the second open reading frame of (i); and

(iii) a sequence encoding an untranslated RNA molecule, or complement thereof;

wherein said second nucleotide sequence is operably linked to a promoter which is recognized by said non-cytopathic, temperature-sensitive alphaviral replicase.

76. The DNA molecule of claim 75, which comprises one second nucleotide sequence.

77. The DNA molecule of claim 75, wherein said second open reading frame is in a translatable format after one RNA-dependent RNA replication event.

78. The DNA molecule of claim 75, wherein said second open reading frame is in a translatable format after three RNA-dependent RNA replication events.

81. The DNA molecule of claim 75, wherein said alphaviral replicase is derived from a Sindbis virus.

82. The DNA molecule of claim 75 which encodes an alphaviral replicase having replicase activity at 34°C which is at least five fold lower than the replicase activity exhibited at 29°C.

83. The DNA molecule of claim 75, wherein the second open reading frame encodes a cytokine, a lymphokine, a tumor necrosis factor, an interferon, a toxic protein, or a prodrug converting enzyme.

84. The DNA molecule of claim 75, wherein the second open reading frame encodes human erythropoietin or human β-interferon.

86. A method of making a recombinant host cell comprising introducing the DNA molecule of claim 75 into a host cell *in vitro*.

87. An *in vitro* cell culture comprising a recombinant host cell produced by the method of claim 86.

88. An *in vitro* cell culture comprising a recombinant host cell comprising the DNA molecule of claim 75.

89. The cell culture of claim 88, wherein some or all of the DNA molecule is stably maintained in said host cell.

90. An RNA molecule transcribed from the DNA molecule of claim 75.

91. An alphaviral particle containing the RNA molecule of claim 90.

92. An *in vitro* cell culture comprising a recombinant host cell comprising the RNA molecule of claim 90.

93. A method for producing a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) introducing at least one DNA molecule of claim 75 into said host cells *in vitro*;

(b) culturing said host cells under conditions suitable for expression of said protein or untranslated RNA molecule; and

(c) recovering said protein or untranslated RNA molecule;

wherein said protein or untranslated RNA molecule is encoded by said DNA molecule.

94. A method for producing a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) introducing at least one RNA molecule of claim 90 into said host cells in vitro;

(b) culturing said host cells under conditions suitable for expression of said protein or untranslated RNA molecule; and

(c) recovering said protein or untranslated RNA molecule;

wherein said protein or untranslated RNA molecule is encoded by said RNA molecule.

95. The method of claim 94, wherein the protein is erythropoietin.

96. The method of claim 94, wherein said RNA is packaged into an alphaviral particle.

97. A method for producing alphaviral particles, said method comprising:

(a) introducing into a host cell *in vitro* at least one DNA molecule of claim 75 having one or more open reading frames which encode alphaviral structural proteins;

(b) growing host cells under culture conditions suitable for the production of alphaviral particles which contain an RNA transcription product of said DNA molecule; and

(c) recovering said alphaviral particles.

98. A method for producing a protein encoded by RNA contained in an alphaviral particle produced by the method of claim 97 in a recombinant host cell comprising:

(a) infecting a host cell *in vitro* with the alphaviral particle;

(b) growing said host cell under culture conditions suitable for the production of said protein; and

(c) recovering said protein.

99. The method of claim 98, wherein said protein is erythropoietin.

100. A method for regulating the expression of a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one DNA molecule of claim 75 into said host cells *in vitro*; and

(c) changing the temperature of the host cell culture from:

(i) a permissive temperature to a restrictive temperature, or

(ii) a restrictive temperature to a permissive temperature;

wherein said protein or untranslated RNA molecule is encoded by said DNA molecule.

101. A method for regulating the expression of a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one RNA molecule of claim 90 into said host cells *in vitro*; and

(c) changing the temperature of the host cell culture from:

(i) a permissive temperature to a restrictive temperature, or

(ii) a restrictive temperature to a permissive temperature;

wherein said protein or untranslated RNA molecule is encoded by said RNA molecule.

103. A DNA vector system comprising one or more polynucleotides which encode RNA molecules, said RNA molecules comprising:

(a) at least one *cis*-acting sequence element,

(b) a first open reading frame having a nucleotide sequence encoding a non-cytopathic, temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of said replicase, and

(c) at least one second nucleotide sequence selected from the group consisting of:

(i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a translatable format after one or more RNA-dependent RNA replication events;

(ii) a sequence complementary to all or part of the second open reading frame of (i); and

(iii) a sequence encoding an untranslated RNA molecule, or complement thereof;

wherein said second nucleotide sequence is operably linked to a promoter which is recognized by said non-cytopathic, temperature-sensitive alphaviral replicase.

105. The DNA vector system of claim 103 which encodes an alphaviral replicase having replicase activity at 34°C which is at least five fold lower than the replicase activity exhibited at 29°C.

106. The DNA vector system of claim 103, wherein the second open reading frame encodes a cytokine, a lymphokine, a tumor necrosis factor, an interferon, a toxic protein, or a prodrug converting enzyme.

107. The DNA vector system of claim 103, wherein the second open reading frame encodes human erythropoietin or human β-interferon.

109. A method of making a recombinant host cell comprising introducing at least one polynucleotide of claim 103 into a host cell *in vitro*.

110. An *in vitro* cell culture comprising a recombinant host cell produced by the method of claim 109.

111. An *in vitro* cell culture comprising a recombinant host cell comprising at least one polynucleotide of claim 103.

112. The cell culture of claim 111, wherein some or all of the polynucleotide sequences of claim 103 are stably maintained in said host cell.

113. A composition comprising one or more RNA molecules transcribed from one or more polynucleotides of the vector system of claim 103.

114. An alphaviral particle containing at least one RNA molecule of claim 113.

115. An *in vitro* cell culture comprising a recombinant host cell comprising at least one RNA molecule of claim 113.

116. A method for producing a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one DNA molecule of claim 103 into said host cells *in vitro*;

(c) recovering said protein or untranslated RNA molecule;

wherein said protein or untranslated RNA molecule is encoded by said DNA molecule.

117. A method for producing a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one RNA molecule of claim 113 into said host cells *in vitro*; and

(c) recovering said protein or untranslated RNA molecule;

wherein said protein or untranslated RNA molecule is encoded by said RNA molecule.

118. The method of claim 117, wherein the protein is erythropoietin.

119. The method of claim 117, wherein said RNA is packaged into an alphaviral particle.

120. A method for producing an alphaviral particle comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing into said host cells *in vitro* at least one DNA molecule of claim 103 having one or more open reading frames which encode alphaviral structural proteins;

(c) producing an alphaviral particle; and

(d) recovering said alphaviral particle.

121. A method for producing a protein in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) infecting said host cells *in vitro* with an alphaviral particle produced by the method of claim 120; and

(c) recovering said protein;

wherein said protein is encoded by nucleic acid contained in said alphaviral particle.

122. The method of claim 121, wherein said protein is erythropoietin.

123. A method for regulating the expression of a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one DNA molecule of claim 103 into said host cells *in vitro*; and

(c) changing the temperature of the host cell culture from:

(i) a permissive temperature to a restrictive temperature, or

(ii) a restrictive temperature to a permissive temperature;

wherein said protein or untranslated RNA molecule is encoded by said DNA molecule.

124. A method for regulating the expression of a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one RNA molecule of claim 111 into said host cells *in vitro*; and

(c) changing the temperature of the host cell culture from:

(i) a permissive temperature to a restrictive temperature, or

(ii) a restrictive temperature to a permissive temperature;

wherein said protein or untranslated RNA molecule is encoded by said RNA molecule.

125. A composition comprising one or more RNA molecules, said RNA molecules comprising:

(a) at least one *cis*-acting sequence element,

(b) a first open reading frame having a nucleotide sequence encoding a non-cytopathic, temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of said replicase, and

(c) at least one second nucleotide sequence selected from the group consisting of:

(i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a

translatable format after one or more RNA-dependent RNA replication events;

(ii) a sequence complementary to all or part of the second open reading frame of (i); and

(iii) a sequence encoding an untranslated RNA molecule, or complement thereof;

wherein said second nucleotide sequence is operably linked to a promoter which is activated by said non-cytopathic, temperature-sensitive alphaviral replicase.

126. The DNA molecule of claim 75, wherein said alphaviral replicase is derived from a Semliki Forest Virus.

127. The DNA molecule of claim 75, wherein said alphaviral replicase is derived from an Aura virus.

128. The DNA molecule of claim 75, wherein said alphaviral replicase is derived from a virus selected from the group consisting of Bebaru virus, Cabassou virus, Chikungunya virus, Eastern equine encephalomyelitis virus, Fort Morgan virus, Getah virus, Kyzylagach virus, Mayoaro virus, Middleburg virus, Mucambo virus, Ndumu virus, Pixuna virus, Tonate virus, Triniti virus, Una virus, Western equine encephalomyelitis virus, Whataroa virus, Venezuelan equine encephalomyelitis virus (VEE), and Ross River virus.

129. The DNA vector system of claim 103, wherein said alphaviral replicase is derived from a Sindbis virus.

130. The DNA vector system of claim 103, said alphaviral replicase is derived from a Semliki Forest Virus.

131. The DNA vector system of claim 103, said alphaviral replicase is derived from an Aura virus.

132. The DNA vector system of claim 103, wherein said alphaviral replicase is derived from a virus selected from the group consisting of Bebaru virus, Cabassou virus, Chikungunya virus, Eastern equine encephalomyelitis virus, Fort Morgan virus, Getah virus, Kyzylagach virus, Mayoaro virus, Middleburg virus, Mucambo virus, Ndumu virus, Pixuna virus, Tonate virus, Triniti virus, Una virus, Western equine encephalomyelitis virus, Whataroa virus, Venezuelan equine encephalomyelitis virus (VEE), and Ross River virus.

133. The RNA molecule of claim 125, wherein said alphaviral replicase is derived from a Sindbis virus.

134. The RNA molecule of claim 125, wherein said alphaviral replicase is derived from a Semliki Forest Virus.

135. The RNA molecule of claim 125, wherein said alphaviral replicase is derived from an Aura virus.

136. The RNA molecule of claim 125, wherein said alphaviral replicase is derived from a virus selected from the group consisting of Bebaru virus, Cabassou virus, Chikungunya virus, Eastern equine encephalomyelitis virus, Fort Morgan virus, Getah virus, Kyzylagach virus, Mayoaro virus, Middleburg virus, Mucambo virus, Ndumu

virus, Pixuna virus, Tonate virus, Triniti virus, Una virus, Western equine encephalomyelitis virus, Whataroa virus, Venezuelan equine encephalomyelitis virus (VEE), and Ross River virus.

137. The DNA molecule of claim 75, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase.

138. The DNA molecule of claim 75, wherein temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

139. The DNA molecule of claim 75, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase, and temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

140. The DNA vector system of claim 103, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase.

141. The DNA vector system of claim 103, wherein temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

142. The DNA vector system of claim 103, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase, and temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

143. The composition of claim 125, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase.

144. The composition of claim 125, wherein temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

145. The composition of claim 125, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase, and temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

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Chapter 9

METHODS FOR GENERATING MULTIPLE SITE-DIRECTED MUTATIONS *IN VITRO*

Jörg Stappert

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I. INTRODUCTION

A. STRATEGIES

Site-directed mutagenesis of cloned genes as well as the mutation of cis-regulatory elements is a powerful and rapid technique for their functional analysis. Since PCR was first described, a number of PCR-based methods have been developed for introducing directed mutations in virtually any position into DNA or for joining unrelated sequences together.

In the simplest cases, point mutations, deletions or insertions can be engineered at the priming sites, using mismatched primers also covering appropriate restriction sites to facilitate recloning of the PCR product. In many cases however, restriction sites are not available, and many mutations cannot be introduced into various sites along a relatively long DNA fragment. To overcome the problem of having to have suitable restriction sites in the vicinity of the desired mutation, several methods have been developed, based on one of the following strategies:

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1. In the first strategy the entire plasmid is amplified using a pair of primers located back to back (e.g., inverse PCR, IPCR; enzymatic inverse PCR, EIPCR), or two sets of primers are used to amplify two overlapping parts of the plasmid which, after annealing via their short complementary DNA stretches, build up the entire plasmid again (e.g., recombinant circle PCR, RCPCR).
2. The second strategy leads to the amplification of a defined mutagenized fragment, which then has to be recloned. The desired mutations are generated by using two primer sets resulting in overlapping PCR fragments or by joining the mutated primers to a "tagged" primer, which enables the selective amplification of the mutated DNA strand.

Following the concept guidelines of this book, an illustrative summary of some of the currently published techniques will be given, their advantages and disadvantages will be pointed out, and finally, in Section II, protocols for RCPCR and tagged PCR will be described, representing each of the strategies mentioned. For a detailed description of the other methods summarized in this chapter, the reader is referred to the references cited for each procedure.

B. SITE-DIRECTED MUTAGENESIS BY AMPLIFICATION OF THE ENTIRE PLASMID

As mentioned, earlier, to do site-directed mutagenesis of cloned DNA by amplifying the whole plasmid rather than just a fragment, four different methods have been described:

1. Inverse PCR mutagenesis (IPCR)[1]
2. Enzymatic inverse PCR mutagenesis (EIPCR)[2]
3. Recombinant circle PCR (RCPCR)[3]
4. Recombination PCR (RPCR)[4]

These four methods have been derived from the originally published inverse PCR.[5] In this technique, two primers that are located back to back on the opposing DNA strands of a plasmid drive the PCR. The resultant PCR product is a linear DNA molecule identical in length to the starting plasmid. Because all these procedures rely on PCR amplification of the entire plasmid, it is not necessary to prepare an appropriate vector fragment or a single-stranded DNA template.

EIPCR is a technique that combines strategies of inverse PCR with the class 2s restriction site approach of Tomic et al.[6] (see Figure 1A). The key step to EIPCR is the incorporation of identical class 2s restriction sites in the primer set used for PCR. Class 2s restriction enzymes have a recognition site located 5′ of the cut site (e.g., Bsp MI *ACCTGC* NNNN*NNNN*). Thus, after completing PCR, the ends of the full-length linearized plasmid are digested with the class 2s enzyme incorporated into the primers. Due to the distance between recognition and cut site, all sequences upstream of the cut site will be lost. Thus in the ligation the only part that becomes part of the plasmid is the *NNNN* overhang, which can be made to be the native DNA sequence. Mutations can be placed into one or both primers and at any location between the enzyme cut site and the exact 3′ match, which should be of a magnitude >15 bp. Since several 2s restriction enzymes have been described, it should generally be possible to design primers containing an appropriate 2s restriction site. The number of positive clones carrying the desired mutation is more than 95%, which makes EIPCR the most efficient method.

In summary, EIPCR has two major advantages: (1) the high percentage of correct clones and (2) the requirement of only one pair of primers to generate a mutation.

In contrast to EIPCR, two different sets of primers are needed for RCPCR and RPCR.[7] In both methods, the product of one inverse PCR is mixed with the product of a second inverse PCR, which is primed at a different location on the same template. Using RCPCR, these products are combined, denatured, and reannealed *in vitro* before competent *Escherichia coli*

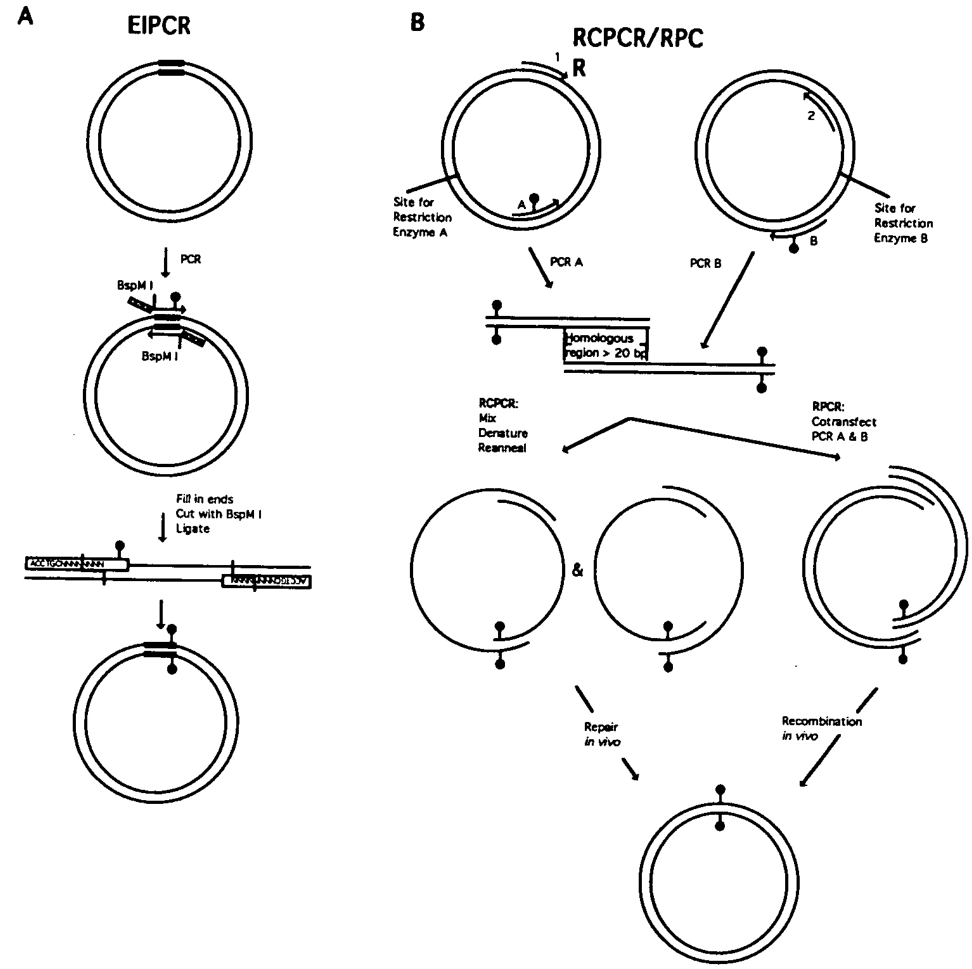

Figure 1. The general scheme for EIPCR (A) and RCPCR/RPCR (B). Double-stranded circles represent double-stranded plasmids; hatched boxes mark 5′ add-on sequences; Solid circles indicate mismatches in the primers and resulting mutations in the PCR products.

are transformed with this construct, as shown in Figure 1B. In contrast to RCPCR, this cross-annealing step is omitted when using RPCR. An equal amount of both PCR reactions is transformed into competent *E. coli*. Upon transformation, the DNA ends undergo homologous recombination *in vivo*, resulting in a circular plasmid. The cross-annealing of the two amplified fragments is mediated by homologous DNA sequences at the 5′ and 3′ and respectively. Whereas the length of homology between mutated ends should be at least 30 bp, the overlapping homology between nonmutated ends depend on the location of primer 1 and 2. Interestingly, it was shown that the number of mutated clones is nearly 100% when using a 22-bp overlap at the nonmutated ends in combination with RPCR, but drops down to 50% when generating the mutation with RCPCR. An inverse correlation was found when increasing the length of homology between nonmutated ends.

All three methods have certain advantages and limitations. All three are rapid and efficient. Nevertheless, the size limit for the template to be amplified is in the range of 5 kb, although mutagenesis of a 7.1-kb construct has been described.[8] A second disadvantage, is the necessity of using relatively long primers; the primers used in these different approaches have to be at least 25 to 30 bp in length. Third, one has to consider the fidelity of the enzyme used for PCR.

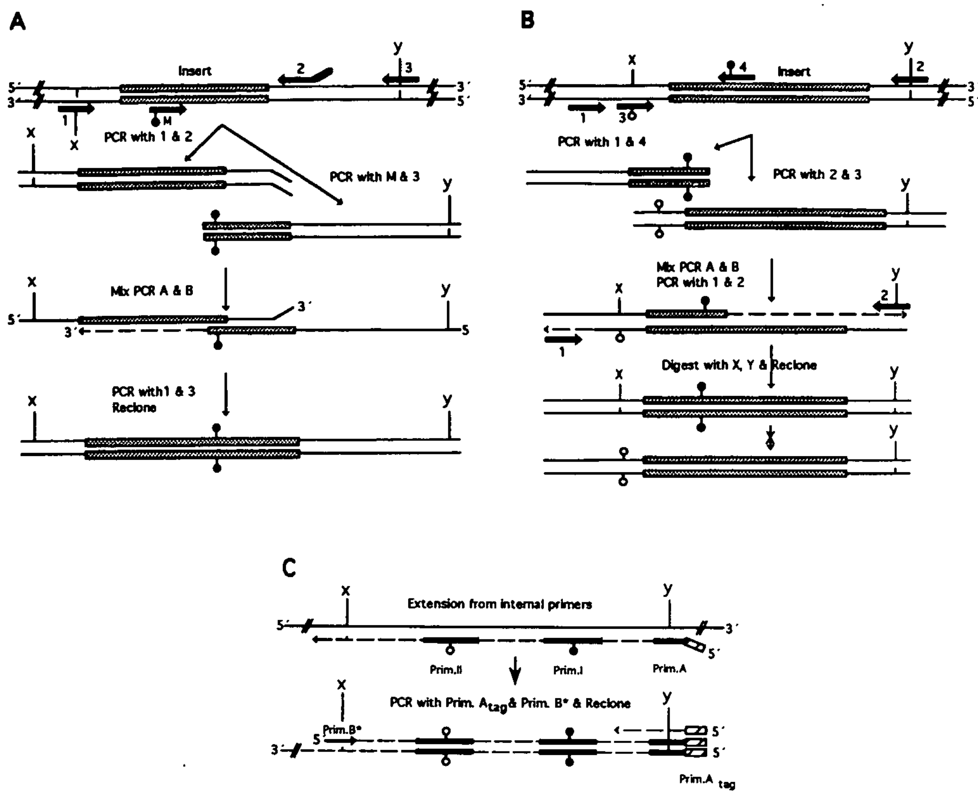

Figure 2. Schematic diagram of three methods used to mutate fragments of a defined length. Primers are represented by black bars; corresponding mismatches are indicated by open and solid circles; Restriction sites are designated X and Y. A detailed description of each method is given in the text.

Since PCR has a low but detectable rate of mutagenesis, polymerases should be used that have a 3′ to 5′ proofreading capability, as this lowers the rate of unwanted mutation.

C. SITE-DIRECTED MUTAGENESIS BY AMPLIFICATION OF SHORT DNA FRAGMENTS

The problem of unwanted PCR errors will be diminished when amplifying only small fragments; this can be achieved by using one of the methods shown in Figure 2.

The two procedures schematically depicted in Figure 2A and 2B represent modifications of the overlap extension method originally published by two groups.[9,10] This method is based on the amplification of two fragments with overlapping ends in which the same mutations are introduced. These fragments are combined and reannealed to each other, and the 3′ overlap of each DNA strand serves as a primer for the 3′ extension of the complementary strand. One disadvantage of the original method is that it requires two new primers for each mutation, limiting one in making an extensive mutagenesis in the same DNA sequence.

The problem has been circumvented by a method described by Mikaelian and Sergeant,[11] as shown in Figure 2A. It requires three universal primers chosen in the vector and only one specific primer for each mutation. As shown, the first step consists of two different rounds of PCR. The two reactions are done using the primer combinations 1,2 and M,3. Primer 1,2 and 3 are homologous to the vector sequence, but primer 2 also contains a mismatched 3′ end; primer M contains the mutation to be introduced into the template DNA. The two amplified fragments are purified, mixed, and subjected to a second PCR with external primers 1 and 3.

During this second PCR, only the mutated DNA strand is amplified, since the 3′ add-on end of primer 2 inhibits the extension of the nonmutated DNA strand. The amplified fragment can be then digested with the appropriate restriction enzymes. The efficiency of this procedure reaches 90%.

A similar approach was used by Ito et al.[12] (Figure 2B). To reclone only the mutated DNA strand, they developed a method called MR (an abbreviation of "modification of a restriction site"). As in the previous technique, three primers are selected as common primers so that each mutation requires only one additional primer. Due to the design of the commonly used primers, it is necessary to reclone the insert to be mutated into a polylinker site of an appropriate plasmid. Even if a suitable restriction site does not exist in the target DNA, it is very easy to create a proper site at both ends of the target DNA by using primers carrying 5′ add-on sequences. As shown in the diagram, primers 1 and 2 are complementary to the neighboring sequences of a polylinker site. Primer 3 is complementary to the polylinker sequence located downstream of primer 1 and has a mismatched nucleotide (nt) to destroy a restriction site. These three primers can be commonly used in a series of different mutagenesis. The fourth primer, however, carries the mutation of interest. By using the primer combinations 1,4 and 2,3, two fragments are amplified, mixed, denatured, and annealed. The resulting products are further amplified by PCR using the external primers 1 and 2, and digested with two different restriction enzymes, X and Y. Although two kinds of DNA are amplified in the second PCR, only the mutated DNA fragment will be recloned, since the restriction site X has been deleted in the nonmutated DNA fragment. Depending on the oligonucleotides used in this technique, the efficiency of getting positive clones can be as high as 100%.

Due to this high efficiency, the described methods are very useful for introducing several mutations into various sites of the inserted DNA. Nevertheless, each new mutation makes it necessary to run a further PCR. Additionally, each newly mutated DNA fragment has to be sequenced in order to detect nucleotide misincorporation generated during DNA amplification.

To avoid this problem, we have developed a method that allows the introduction of several mutations in only one step.[13] To do this we combined extension of mutated internal primers by T4 DNA polymerase with selective amplification of the mutated DNA strand by PCR from added-on external primers. The principle of this method is summarized in Figure 2C.

In the first step, two or even more mutagenic primers (I, II) and Primer A are annealed to the template. Primer A has a suitable restriction site and a 5′ add-on sequence that is not complementary to the template. After primer extension and ligation reaction, the mutated DNA strand is selectively amplified by PCR in the second step by using the two outer primers, A_{tag} and B*, as common primers. Each new mutation thus requires only one additional primer. The percentage of positive clones carrying both mutations depends on whether ss or ds DNA is used as a template. When using ss DNA, up to 80% of all clones will be positive for two mutations, in contrast to 40 to 50% when using ds plasmid DNA. For this method, bacteriophage T4 DNA polymerase or Sequenase should be used in the polymerase extension reaction. Unlike the Klenow fragment of *E. coli* DNA polymerase I, neither of these enzymes is able to displace the mutagenic oligonucleotide from its template. Therefore there should be no limit to the number of different mutagenic primers that can be used in a single *in vitro* site-directed mutagenic reaction. Indeed, as has been reported by Perlack,[15] using the classical procedure of Kunkel,[14] 14% of the clones tested were positive for seven of seven different mutations generated in one single step. This is the same efficiency one would expect when using the PCR-based method.

In the protocols described below, practical approaches concerning the generation of mutations are given exclusively for the mutational step itself. Commonly used techniques like plasmid preparation, ligation, transformation of competent *E. coli*, etc., should be done using accepted protocols.[16,17]

II. MATERIALS AND METHODS

A. MATERIALS

Plasmids used as PCR templates as well as for DNA sequencing were prepared with Qiagen mini- or midi-columns (Qiagen, Düsseldorf). Sequence analysis of the mutants was done on double-stranded DNA, using the T7 Sequenase Kit (U.S. Biochemical) according to the manufacturer's description. All oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and then purified using a C-18 reversed-phase high-pressure liquid chromatography (HPLC) column. Except the *Taq* DNA polymerase, which was purchased from Amersham Buchler (Braunschweig), all restriction and modification enzymes were purchased from GIBCO BRL (Eggenstein) or from Boehringer Mannheim.

B. RECOMBINANT CIRCLE PCR (RCPCR)

1. Primer Design

RCPCR requires a total of four primers per mutagenesis reaction. If the insert to be mutated has been cloned into a polylinker site of a vector, only two new primers need to be synthesized per mutagenesis reaction. Since the nonmutating primers will be located outside the mutagenesis region, they can be reused to mutate any given region of the insert. The nonmutating primers should be at least 15 nt in length, where the mutating primers have an overall length of >25 nt carrying at least 15 nt of exact complementarity to each other at their 5′ ends. It is also possible to use mutating primers with an exact complementarity. The homology length between nonmutated ends will greatly influence the efficiency of this method.

2. PCR Amplification

Before PCR, each plasmid has to be linearized by restriction enzyme digestion outside the region to be amplified. In each of the two separate PCR amplifications for a given mutagenesis, we set up the following reaction mixture in 100 μl: 10 ng of pBlueskript SKII$^+$ DNA containing the insert to be mutated, each primer at 1 μ*M*, 100 μ*M* of each dNTP, 2.5 U *Taq* polymerase in Taq-Buffer (10 × Taq-Buffer is 100 m*M* Tris-HCl, pH 8.4; 500 m*M* NaCl; 20 m*M* $MgCl_2$; 1 mg/ml gelatin). Prior to amplification, 60 μl of mineral oil is placed on top of each reaction mixture. For the amplification, reactants are subjected to 30 c of PCR with the following parameters: denaturation at 95°C for 0.5 min; annealing at 50°C for 0.5 min; and extension at 72°C for 2 min.

3. Purification of the PCR Product

As published by Jones and Winistorfer,[7] there is no need to further purify the PCR products. To remove mineral oil, we recommend freezing the probe. Alternatively, each PCR product may be carefully removed with a thin micropipet and transferred into a new reaction tube.

4. Annealing of the PCR Products

In order to generate recombinant circles *in vitro*, equal amounts of the two PCR products have to be combined, denatured at 95°C for 5 min, reannealed at 50°C for 2 h, and returned to room temperature prior to transfection.

5. Transformation of *E. coli* and Screening of Colonies

Competent *E. coli* should be transformed with 2 to 5 μl of combined PCR product. In cases of restriction site deletions or insertions, positive clones can be identified either by preparation of the plasmid DNA or by using a PCR-based procedure described by Güssow and Clackson.[18] Briefly: Colonies are resuspended in 0.5 ml of water (live bacteria can be rescued at this stage) and boiled in a water bath for 5 min. After centrifugation for 2 min at 13 to 16,000 *g*, 5 μl of the supernatant is subjected to 30 c of PCR amplification with primers that flank the mutagen-

esis site. The orientation of the insert may be screened using a third primer within the insert. Alternatively 1/10 of the unpurified PCR product may be digested by the appropriate restriction enzyme

C. TAGGED PCR MUTAGENESIS METHOD

1. Primer Design

In order to use Primers A and B* together, they should be complementary to vector sequences flanking the insert to be mutated. Primer A should have a length of >30 nt and contain 15 nt at the 5′ end, which are not homologous to the template. A suitable restriction site should be available within the homologous region. Primer A_{tag}, which is identical to the 5′ add-on sequence of primer A, should have a GC content of >50%. Primer B* has to cover a second restriction site and should have a minimum length of 15 nt. The mutagenic primers we use are, respectively, 18 to 21 nt in length and contain up to four mismatches relative to the template DNA. A total of 6 nt exact homology to the template is included on each end to ensure proper hybridization.

2. 5′ Phosphorylation of the Mutating Primers

Before starting primer extension, the mutating primers have to be phosphorylated with Kinase in order to allow ligation of its 5′ ends. Set up the following reaction mixture in 20 µl: 200 pmol primer, 1 × Kinase Buffer (10 × Kinase Buffer is 0.5 *M* Tris-HCl, pH 8.0; 0.1 *M* $MgCl_2$), 10 µ*M* DTT, 2 m*M* ATP, and 5 U polynucleotide kinase. Incubate at 37°C for 30 min and then at 65°C for 10 min to inactivate the kinase. Dilute the phosphorylated oligonucleotides to a final concentration of 2 pmol/µl in water.

3. Denaturation of the ds Template DNA

In order to facilitate annealing of the primers to the template, it is necessary to denature the ds DNA. This can be done either by heat or alkaline denaturation. In both procedures, use 0.2 to 0.4 pmol ds template DNA. If using alkaline denaturation, incubate the template DNA in a reaction volume of 20 µl, together with 0.2 *M* NaOH, at room temperature for 5 min. Neutralize the mixture by adding 8 µl 5 *M* ammonium acetate, pH 7.4. Precipitate the DNA with 100 µl ethanol at –70°C for 5 min. Centrifuge at 16,000 *g* for 5 min, then wash with 70% ethanol. Dry at room temperature for 10 min.

4. Annealing and Extension Reaction

To anneal primers to the denatured template DNA, incubate 5 to 10 × excess of phosphorylated primers (mutagenized primers and primer A) in 1 × Annealing Buffer (10 × Annealing Buffer is 200 m*M* Tris-HCl, pH 7.4; 20 m*M* $MgCl_2$; 500 m*M* NaCl) in a total volume of 10 µl. Heat to 65°C for 3 min, and allow to cool slowly to room temperature for 30 min.

Synthesis of the complementary DNA strand is done in a volume of 20 µl containing the same annealing mixture plus 2.5 U T4 DNA polymerase, 1 U T4 DNA ligase, and 1 × Synthesis Buffer (10 × Synthesis Buffer is 5 m*M* of each dNTP; 10 m*M* ATP; 100 m*M* Tris-HCl, pH 8.0; 10 m*M* EDTA). After incubating at 37°C for 90 min, add 10 m*M* Tris-HCl; 10 m*M* EDTA, pH 8.0 (final concentration), and stop the reaction by freezing.

5. PCR Amplification

For amplification of the mutated DNA strand use the external primers, A_{tag} and B*, at 1 µ*M* with 1/5 volume of the crude "extension" reaction, 100 µ*M* of each dNTP, and 2.5 U *Taq* polymerase in Taq-Buffer (for 10 × Taq-Buffer, see RCPCR) in a reaction volume of 100 µl. Before amplification, overlay the mixture with 60 µl of mineral oil. Then subject the PCR mixture to 30 c of amplification: 0.5 min at 95°C; 0.5 min at 55°C; 1 min at 72°C. After removing the mineral oil, 5 µl of the PCR mixture can be analyzed on a 1% TBE agarose gel

and stained with EtBr. To determine the efficiency of incorporation of the mutated primers, 5 μl of each reaction should be digested with the appropriate restriction enzymes. Before recloning, we recommend purifying the PCR products. Clones can be analyzed as described above.

III. DISCUSSION

As mentioned above, the efficiency of the RCPCR method is influenced by the length of homology between nonmutated ends. Whereas 50% of all clones are positive when containing a 25-bp stretch of homology, up to 90% of the clones will have the mutation if the length of homology is increased to 2800 bp. In order to reduce background transformations by the original nonmutated PCR template, one must use linearized PCR templates, which have been cut outside the region to be amplified.

One big advantage of RCPCR, as well as of all other methods using the mutated primers to amplify the entire plasmid for amplification, is the fact that cloning steps are omitted. Nevertheless, for each mutation it is necessary to do a new round of amplification. Site-directed mutagenesis using the tagged PCR method is therefore the method of choice if several mutations have to be generated.

The possibility of simultaneously introducing several mutations in a single step makes this method much faster than the others as long as suitable "markers" can be introduced in each mutation. For that reason it is necessary to use mutagenic oligonucleotides carrying the desired mutation together with a second nt mismatch. This second, "silent" mutation is necessary to generate or delete a restriction site within the primer sequence in order to facilitate screening for positive clones. If such markers are absent, the method becomes more elaborate because all colonies have to be hybridized using the primers as probes. On the other hand, this problem will still occur in all methods not approaching a mutation efficiency of 100%.

For this reason, it is necessary in each case to choose the *in vitro* mutagenesis method appropriate for a given problem.

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PCR TECHNOLOGY
Current Innovations

Edited by

Hugh G. Griffin
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CRC Press
Boca Raton Ann Arbor London Tokyo

Library of Congress Cataloging-in-Publication Data

PCR technology : current innovations / edited by Hugh G. Griffin,
Annette M. Griffin.
p. cm.
Includes bibliographical references and index.
ISBN 0-8493-8674-8
1. Polymerase chain reaction—Methodology. I. Griffin, Hugh G.
II. Griffin, Annette M.
QP606.D46P362 1994
574.87'3282—dc20

93-37561
CIP

International Standard Book Number 0-8493-8674-8
Library of Congress Card Number 93-37561
Printed in the United States of America 3 4 5 6 7 8 9 0
Printed on acid-free paper

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 1349
RENNER *et al.*	Art Unit: 1635
Appl. No. 09/275,883	Examiner: Schnizer, R.
Filed: March 25, 1999	Atty. Docket: 1700.0020001/JAG/FRC
For: **Inducible Alphaviral Gene Expression System**	

Brief on Appeal Under 37 C.F.R. § 1.192

Mail Stop Appeal Brief - Patents

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

A Notice of Appeal from the rejection of claims 75-78, 81-84, 86-101, 103, 105-107 and 109-145 (twice rejected) was filed on December 17, 2003. Appellants hereby file this Appeal Brief in triplicate, together with the required brief filing fee.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

Table of Contents

I. Real Party in Interest (37 C.F.R. § 1.192(c)(1))

The real party in interest in this appeal is Cytos Biotechnology AG.

II. Related Appeals and Interferences (37 C.F.R. § 1.192(c)(2))

Appellants' undersigned representative is not aware of any appeals or interferences related to this application.

III. Status of Claims (37 C.F.R. § 1.192(c)(3))

The present application was filed on March 25, 1999. As originally filed, the application contained a total of 74 claims.

In an Amendment filed November 15, 2000, claims 1-74 were cancelled, and claims 75-125 were added.

In an Amendment filed July 31, 2001, claims 80, 85, 104 and 108 were cancelled, claims 75, 81, 103 and 125 were amended, and claims 126-136 were added.

In an Amendment filed November 4, 2002, claim 79 was cancelled, claims 75, 81, 82, 86, 93, 94, 97, 98, 100, 101, 103, 105, 109, 116, 117, 120, 121, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135 and 136 were amended, and claims 137-145 were added.

Claims 75-78, 81-84, 86-103, 105-107 and 109-145 are pending in the application. Claim 102 is allowed. Claims 75-78, 81-84, 86-101, 103, 105-107 and 109-145 are now on appeal. A copy of the claims on appeal can be found in the attached Appendix.

IV. Status of Amendments (37 C.F.R. § 1.192(c)(4))

All amendments have been entered. No amendments have been filed subsequent to the issuance of the Office Action dated June 17, 2003.

V. Summary of Invention (37 C.F.R. § 1.192(c)(5))

A. Background

Alphaviruses have a positive-sense RNA genome. The 5' two-thirds of the alphaviral genome encodes four nonstructural proteins, nsP1 through nsP4. The nonstructural proteins together make up the alphaviral replicase which replicates the alphaviral genome in the cytoplasm of infected host cells. The 3' one-third of the genome encodes the structural proteins which form the capsid and envelope protein that encloses the alphaviral genome.

Investigators have modified the alphaviral genome to allow high levels of transgene expression. The modified alphaviral genome, known as a replicon, is an RNA molecule comprising the replicase-encoding region (nsPs1-4) and at least one *cis*-acting sequence element to which the replicase binds to initiate RNA replication. The region encoding the structural proteins is replaced with a heterologous sequence or gene of interest.

Replicons are introduced into host cells. In the cytoplasm of the host cell, a complementary negative-strand RNA molecule is synthesized from the replicon by the alphaviral replicase. The negative strand in turn serves as the template for two distinct positive-strand RNA species. One species corresponds to the entire replicon. The second RNA species corresponds to the 3' region of the replicon encoding the heterologous sequence or gene of interest. The second RNA species is known as the

subgenomic RNA species. The gene product of interst is translated from the subgenomic RNA species.

Originally, alphaviral replicons (RNA molecules) were transcribed *in vitro* before being introduced into host cells. More recently, DNA-based alphaviral vectors (plasmids) have been developed. DNA-based alphaviral vectors are transfected into host cells. The replicon is transcribed from the DNA in the nucleus of the host cell and is subsequently transported into the cytoplasm. Replication and transgene expression from the replicon take place in the cytoplasm.

Alphaviral replicases, however, are lethal to cells. Gene expression from traditional alphaviral vectors (including RNA-based and DNA based vectors) is therefore only transient.

B. The Present Invention

The present invention avoids the cell lethality problems that are encountered with traditional alphaviral expression vectors. The present invention overcomes the cell lethality problems by taking advantage of mutations in the alphaviral genome which render the alphaviral replicase non-cytopathic and temperature sensitive. The alphaviral replicases encoded from the vectors of the present invention are not lethal to cells and only function at certain temperatures. These characteristics allow for much greater transgene expression than is possible with the alphaviral vectors previously known in the art.

More specifically, the present invention is directed to DNA molecules and vector systems which encode RNA molecules comprising: (a) at least one *cis*-acting sequence

element, (b) a first open reading frame which encodes a non-cytopathic temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of said replicase, and (c) at least one second nucleotide sequence selected from the group consisting of: (i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a translatable format after one or more RNA-dependent RNA replication events; (ii) a sequence complementary to all or part of the second open reading frame of (i); and (iii) a sequence encoding an untranslated RNA molecule, or complement thereof; wherein said second nucleotide sequence is operably linked to a promoter which is recognized by said non-cytopathic, temperature-sensitive alphaviral replicase (claims 75-78, 81-84, 103, 105-107 and 125-136). Support for these aspects of the invention can be found throughout the specification, for example, at page 7, lines 4-17, and at page 21, line 23, through page 22, line 25.

The invention also includes methods involving the use of the DNA molecules/vector systems of the invention. The invention includes methods of making a recombinant host cell (claims 86 and 109). Support for this aspect of the invention can be found throughout the specification, for example, at page 8, lines 14-15. The invention includes methods for producing a protein or an untranslated RNA molecule (claims 93-96, 98, 99, 116-119, 121 and 122). Support for this aspect of the invention can be found throughout the specification, for example, at page 8, lines 23-27. The invention includes methods for producing alphaviral particles (claims 97 and 120). Support for this aspect of the invention can be found throughout the specification, for example, at page 8, lines 3-7. The invention includes methods for regulating the expression of a protein or an

untranslated RNA molecule in a recombinant host cell (claims 100, 101, 123 and 124). Support for this aspect of the invention can be found throughout the specification, for example, at page 22, line 26, through page 23, line 21.

The invention also includes *in vitro* cell cultures comprising recombinant host cells comprising the DNA molecules of the invention (claims 87-89, 92, 110-112 and 115). Support for this aspect of the invention can be found throughout the specification, for example, at page 29, line 1, through page 30, line 13. The invention also includes RNA molecules transcribed from the DNA molecules of the invention, and compositions comprising such RNA molecules (claims 90 and 113), and alphaviral particles containing RNA molecules transcribed from the DNA molecules of the invention (claims 91 and 114). Support for these aspects of the invention can be found throughout the specification, for example, at page 8, lines 3-7.

The invention also includes the DNA molecules/vector systems of the invention, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of the replicase (claims 137, 140 and 143). The invention also includes the DNA molecules/vector systems of the invention, wherein temperature sensitivity is conferred by one or more mutations in the nsP4 gene of the replicase (claims 138, 141 and 144). The invention also includes the DNA molecules/vector systems of the invention, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of the replicase and temperature sensitivity is conferred by one or more mutations in the nsP4 gene of the replicase (claims 139, 142 and 145). Support for these aspects of the invention can be found throughout the specification, for example, at page 21, line 23, through page 22, line 25.

VI. Issues on Appeal (37 C.F.R. § 1.192(c)(6))

A. Written Description

The first issue on appeal is whether claims 75-78, 81-84, 86-101, 103, 105-107 and 109-145 contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

B. Enablement

The second issue on appeal is whether the specification would have enabled one of ordinary skill in the art to which the invention pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with claims 75-78, 81-84, 86-101, 103, 105-107 and 109-136.

VII. Grouping of Claims (37 C.F.R. § 1.192(c)(7))

For the purpose of this appeal, the claims do not stand or fall together. The claims are grouped as follows:

- Group I: Claims 75-78, 81-84, 86-101, 103, 105-107 and 109-136; and
- Group II: Claims 137-145.

VIII. Argument (37 C.F.R. § 1.192(c)(8))

A. Written Description

1. Legal Standard for Written Description

To satisfy the written description requirement of 35 USC § 112, first paragraph, an Applicant must convey with reasonable clarity to those skilled in the art that, as of the effective filing date, the Applicant was in possession of the invention. *See Vas-Cath,*

Inc. v. Mahurkar, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). As made clear by the Federal Circuit, "[t]he written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [he or she] invented what is claimed.'" *Union Oil Co. of Cal. v. Atlantic Richfield Co.*, 208 F.3d 989, 997, 54 USPQ2d 1227, 1232 (Fed. Cir. 2000); *see also Amgen Inc. v. Hoechst Marion Roussel Inc.*, 65 USPQ2d 1385, 1397 (Fed. Cir. 2003) ("[t]he purpose of the written description requirement is to prevent an applicant from later asserting that he invented that which he did not.")

The Federal Circuit has recently adopted the standard for determining compliance with the written description requirement as set forth in the USPTO's "Guidelines for the Examination of Patent Applications under 35 U.S.C. § 112, first paragraph, Written Description Requirement." *See Enzo Biochem, Inc. v. Gen-Probe Inc.*, 296 F.3d 1316, 1324, 63 USPQ2d 1609, 1613 (Fed. Cir. 2002). According to the USPTO's Guidelines:

> An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.

MPEP § 2163; *See also, Enzo*, 296 F.3d at 1324, 63 USPQ2d at 1613.

2. *The Written Description Requirement Is Fully Satisfied for the Subject Matter of the Claims of Group I (Claims 75-78, 81-84, 86-101, 103, 105-107 and 109-136)*

(a) The USPTO's Written Description Guidelines are Satisfied for the Claims of Group I

(i) The Written Description Requirement May be Satisfied By Disclosure of Structural Characteristics, Functional Characteristics, and a Known or Disclosed Correlation Between Function and Structure

The USPTO's Written Description Guidelines indicate that the written description requirement of 35 U.S.C. § 112, first paragraph, may be satisfied by the disclosure of relevant identifying structural characteristics and/or functional characteristics when there is a known or disclosed correlation between function and structure. *See* MPEP § 2163. As discussed in more detail below, the present specification discloses relevant identifying structural and functional characteristics of the claimed invention. In addition, the claims provide a structural and functional definition of the invention. Furthermore, there is a known correlation between the function of the nucleic acid molecules of the invention and their structure. Therefore, an analysis based on the USPTO's Written Description Guidelines compels the conclusion that the claimed invention is more than adequately described by the specification.

(ii) The Specification Discloses the Structural and Functional Characteristics of the Subject Matter Encompassed by the Claims

The claims of Group I are directed to, or involve the use of, nucleic acid molecules which comprise a first open reading frame which encodes a non-cytopathic, temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the non-

structural proteins of the replicase. The present specification discloses the structural and functional characteristics of the claimed invention.

The specification provides a working example of a nucleic acid molecule that encodes a non-cytopathic, temperature sensitive alphaviral replicase. The construction of this nucleic acid molecule, designated pCYTts, is described in the specification at page 41, line 2, through page 43, line 8 (Example 1). This exemplary nucleic acid molecule encodes a Sindbis virus replicase containing a Pro726Ser mutation in nsP2 and a Gly153Glu mutation in nsP4. It was further demonstrated that pCYTts, containing various heterologous genes, is non-cytopathic and allows temperature sensitive transgene expression. *See* specification at page 43, line 10, through page 51, line 9 (Examples 2-6). The complete nucleotide sequence of pCYTts is set forth in the specification at Figures 3A-3D (SEQ ID NO:1).

Based on the working examples in the specification, a person of ordinary skill in the art would recognize the pertinent functional and structural characteristics of the invention. That is, a person of ordinary skill in the art would recognize that the nucleic acid molecules of the invention encode a temperature sensitive, non-cytopathic replicase, and that non-cytopathicity and temperature sensitivity are conferred by mutations in the nonstructural protein genes of the replicase.

In other places, the specification makes explicit the fact that the invention includes nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases, and that non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of

the replicase. For example, the specification provides the following summary of the invention:

> The present invention provides compositions and methods for regulated expression of proteins or untranslated RNA molecules in recombinant host cells. More specifically, the present invention provides polynucleotides and methods which allow precise regulation of the amount of specific RNA molecules produced in stably transfected recombinant host cells. This precise regulation results from the use of a temperature-sensitive RNA-dependent RNA polymerase (*i.e.*, a replicase) which only replicates RNA molecules, to form new RNA molecules, at permissive temperatures.

See specification at page 6, lines 17-24. It is further noted that:

> When using alphavirus replicase proteins, in most instances, it is desirable to convert the cytopathic phenotype of the replicase protein to a non-cytopathic phenotype. Preferred mutations which confer such a phenotype are in the nsp2 gene (*e.g.*, the proline residue at position 726 is replaced with a serine residue). Mutations are known in the art which render the replicase protein non-cytopathic (Weiss *et al., J. Virol. 33*:463-474 (1980); Dryga *et al., Virology 228*:74-83 (1997)). These mutations may be introduced by a number of means, including site directed mutagenesis.
>
> As noted above, when a non-cytopathic Sindbis virus replicase is used in the practice of the invention, a mutation may be introduced in the nsp2 gene. One such mutation results from the exchange of the proline residue at position 726 to another of the 20 natural occurring amino acids, such as a serine (abbreviated as "Pro 726 Ser"). *Alternatively, any other mutation rendering the replicase molecule non-cytopathic is within the scope of the invention.* The creation and the identification of mutations which render the Sindbis replicase non-cytopathic are described in more detail elsewhere (Weiss *et al., J. Virol. 33*:463-474 (1980); Dryga *et al., Virology 228*:74-83 (1997); patent application WO 97/38087). Further, methods for inducing such mutations are known in the art (*see, e.g.*, Sambrook, J. *et al.*, eds., MOLECULAR CLONING,

> A LABORATORY MANUAL, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel, F. *et al.*, eds., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John H. Wiley & Sons, Inc. (1997)).

See specification at page 21, line 23, through page 22, line 12 (emphasis added).

With respect to temperature sensitivity, it is noted that:

> Temperature sensitivity (*ts*) may be conferred, for example, by the introduction of a mutation in the nsp4 gene of the replicase. Preferably, mutations which confer a temperature-sensitive phenotype upon replicase activities are in a protein in complementation group F (Lemm *et al.*, *J. Virol.* *64*:3001-3011 (1990)). For example, a temperature-sensitive phenotype may be conferred by changing Gly 153 of nsp4 to Glu. *Additionally, any other mutation which renders replicase activity temperature-sensitive can be used in the practice of the invention.* Methods for creating and identifying new temperature-sensitive mutants are described by Pfefferkorn (Burge and Pfefferkorn, *Virol.* *30*:204-213(1966); Burge and Pfefferkorn, *Virol.* *30*:214-223 (1966)). Further, any method useful for producing and identifying *ts* mutants which allow for the temperature-sensitive regulation of replicase activity can be employed to generate and isolate such mutants.

See specification at page 22, lines 13-25 (emphasis added).

Thus, it is clear from the specification that the invention encompasses nucleic acid molecules that encode non-cytopathic, temperature-sensitive alphaviral replicases. It is also clear from the specification that the properties of non-cytopathicity and temperature sensitivity can be conferred upon the replicases by *any mutation(s)* that render the replicase non-cytopathic and temperature sensitive. Methods for obtaining

such mutations were well known to persons of ordinary skill in the art as of the effective filing date of the application. *See* discussion below.

(iii) There is a Known Correlation Between Function (Non-Cytopathic and Temperature Sensitivity) and Structure (One or More Mutations in the Genes Encoding the Nonstructural Proteins of the Replicase)

The correlation between (a) the phenotypes of non-cytopathicity and temperature sensitivity and (b) mutations in the genes encoding the nonstructural proteins of alphaviral replicases was known in the art as of the effective filing date of the present application. Thus, in the context of the present claims, there was a known correlation between function and structure.

There are numerous examples in the scientific literature of mutations in alphaviral nsP genes that cause temperature-sensitivity and/or non-cytopathicity. Examples of such mutations are described in Suopanki *et al.*, *J. Gen. Virol.* *79*:309-319 (1998); LaStarza *et al.*, *J. Virol.* *68*:5781-5791 (1994); Wang *et al.*, *J. Virol.* *65*:985-988 (1991); Shirako and Strauss, *Virology 177*:54-64 (1990); Hardy *et al.*, *Virology 177*:199-208 (1990); Hahn *et al.*, *J. Virol.* *63*:3142-3150 (1989); Sawicki and Sawicki, *J. Virol.* *67*:3605-3610 (1993); Hahn *et al.*, *J. Virol.* *63*:1194-1202 (1989); Shirako and Strauss, *J. Virol.* *72*:2310-2315 (1998); Lemm *et al.*, *J. Virol.* *64*:3001-3011 (1990); Sawicki *et al.*, *Virology 174*:43-52 (1990) (copies submitted with Appellants' Supplemental Reply filed on March 31, 2003, as Exhibits 1-11, respectively); and Dryga *et al.*, *Virology 228*:74-83 (1997) (copy submitted as document AS6 in the Information Disclosure Statement filed on June 28, 1999). Thus, it was recognized in the art that temperature sensitivity and non-cytopathicity were conferred by mutations in the nsP genes of alphaviral replicases.

Additional mutations that render an alphaviral replicase non-cytopathic and temperature sensitive could have easily been obtained by persons of ordinary skill in the art. The ability of those skilled in the art to obtain non-cytopathic, temperature-sensitive alphaviral replicases is described in the Declaration of Dr. Sondra Schlesinger Under 37 C.F.R. § 1.132 (copy submitted with Appellants' Supplemental Reply filed on March 31, 2003) and is also addressed in the response to the Enablement rejection below.

In view of the numerous examples of nucleic acid molecules encoding non-cytopathic and/or temperature sensitive alphaviral replicases having mutations in the non-structural protein genes, and the ease with which one of ordinary skill in the art could have obtained additional non-cytopathic, temperature sensitive alphaviral replicases (*see* section VIII.B.2, below), it must be concluded that there was a known correlation between the function and structure of non-cytopathic, temperature sensitive alphaviral replicases.

(b) The Sindbis Virus Mutations Disclosed in the Specification Would Have Directed Persons of Ordinary Skill in the Art to Additional Non-Cytopathic, Temperature Sensitive Mutations in Other Alphaviruses, Due to the High Degree of Sequence Homology Shared Among Alphaviral Nonstructural Proteins

The working example in the specification involves a Sindbis virus non-cytopathic, temperature-sensitive replicase. The nsPs from various alphaviruses share a high degree of sequence homology. The high level of sequence homology that exists among the nsPs of alphaviruses is illustrated in the table, submitted with Appellants' Supplemental Reply filed on March 31, 2003, as Exhibit 13. This table shows the percent amino acid sequence identity and similarity that exists between the nsPs of the following alphaviruses: O'nyong-nyong virus (ONG), Ross River virus (RRV), Semliki

Forest virus (SFV), Sindbis virus (SinV), Venezuelan equine encephalitis virus (VEEV) and Western equine encephalomyelitis virus (WEEV). Appellants have also submitted individual amino acid sequence alignments comparing the sequences of alphaviral nsP1, nsP2, nsP3 and nsP4 proteins. *See* Appellants' Supplemental Reply filed on March 31, 2003, Exhibits 14, 15, 16 and 17, respectively). Finally, a phylogenetic tree was presented showing the evolutionary relationship among the nsP2 and nsP4 proteins from various alphaviruses. *See* Appellants' Supplemental Reply filed on March 31, 2003, Exhibit 18. Although these sequence comparisons involve amino acid sequences, they necessarily reflect the high degree of correlation that exists among the alphaviral nsP genes at the nucleic acid level.

In view of the high degree of sequence homology that exists among the nsPs of alphaviruses, the exemplary mutations disclosed in the specification would have directed persons of ordinary skill in the art to the nucleotide positions in other alphaviruses that, when mutated, would likely result in non-cytopathicity and temperature sensitivity. Thus, although the specification describes a nucleic acid molecule that encodes a non-cytopathic, temperature sensitive *Sindbis virus* replicase, the exemplary disclosed species would have been regarded by persons of ordinary skill in the art as, in effect, a disclosure of non-cytopathic, temperature sensitive mutations in the nonstructural protein genes of *all* alphaviruses. In view of the exemplary embodiment provided in the specification, and the high degree of sequence homology that exists among alphaviral nsPs, a person of ordinary skill in the art would conclude that Appellants were in possession of the claimed subject matter.

(c) Summary

The written description requirement of 35 U.S.C. § 112, first paragraph is fully satisfied for the subject matter of the claims of Group I. The USPTO's guidelines indicate that the written description requirement may be satisfied by the disclosure of functional and structural characteristics when there is a known or disclosed correlation between function structure. Here, Appellants have disclosed both the functional characteristics (non-cytopathicity and temperature sensitivity) and the structural characteristics (one or more mutations in the genes encoding the nonstructural proteins of the replicase) of the claimed subject matter. It was well known in the art that mutations in alphaviral nonstructural protein genes cause non-cytopathicity and/or temperature sensitivity. Thus, there is a known correlation between function and structure. In addition, the disclosure of an exemplary nucleic acid molecule that encodes a non-cytopathic, temperature sensitive Sindbis virus replicase (pCYTts) would have been regarded by persons of ordinary skill in the art as effectively disclosing non-cytopathic, temperature sensitive mutations in all alphavirus species because of the high level of sequence homology that exists among the nonstructural proteins of alphaviruses. Thus, the written description requirement of 35 U.S.C. § 112, first paragraph, is fully satisfied for the subject matter of the claims of Group I.

3. The Written Description Requirement Is Fully Satisfied for the Subject Matter of the Claims of Group II (Claims 137-145)

The claims of Group II depend from Group I claims 75, 103 and 125. The subject matter of the claims of Group II is adequately described in the specification for at least the same reasons that the subject matter of the claims of Group I is adequately described. *See* discussion in section VIII.A.2, above. In addition, the claims of Group

II specify that non-cytopathicity is conferred by one or more mutations in the nsP2 gene of the replicase, and/or that temperature sensitivity is conferred by one or more mutations in the nsP4 gene of the replicase. Thus, the claims of Group II provide even more structural definition for the nucleic acid molecules encoding the alphaviral replicase than do the claims of Group I. The correlation between structure and function is therefore even more evident for the claims of Group II. In view of the high level of structural definition provided for the nucleic acid molecules in the claims of Group II, a person of ordinary skill in the art would appreciate that Appellants were in possession of the subject matter of these claims. The written description requirement of 35 U.S.C. § 112, first paragraph, is fully satisfied for the claims of Group II.

4. The Examiner Has Not Established a Prima Facie Case of Insufficient Written Description

(a) Satisfaction of the Written Description Requirement Does Not Necessarily Require the Disclosure of Multiple Working Examples

The Examiner has not established a *prima facie* case of insufficient written description. The Examiner has based the written description rejection on the absence of multiple working examples of non-cytopathic, temperature-sensitive alphaviral replicases in the specification. *See* Office Action dated June 17, 2003, page 4, lines 1-4. According to the Examiner, "[t]he central issue in this analysis is whether Applicant has disclosed a number of species which is representative of the claimed genus." *See* Office Action dated June 17, 2003, page 4, lines 1-2. The Examiner has cited the Written Description Guidelines to support this assertion. *See* Office Action dated June 17, 2003, page 3, lines 12-14.

Satisfaction of the written description requirement, however, does not necessarily require the disclosure of multiple working examples. There are other factors that must be taken into consideration such as, *e.g.*, the disclosure of structural and functional characteristics and the correlation between structure and function. *See* MPEP § 2163; *see also Enzo*, 296 F.3d at 1324, 63 USPQ2d at 1613. As noted above, the present specification discloses (a) the functional characteristics of the nucleic acid molecules included in the invention (*i.e.*, that they encode a non-cytopathic, temperature sensitive alphaviral replicase); and (b) the structural characteristics of the nucleic acid molecules included in the invention (*i.e.*, that non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of the replicase). In addition, it was well known in the art that non-cytopathicity and temperature sensitivity are caused by mutations in the genes encoding the nonstructural proteins of alphaviral replicases. *See* section VIII.A.2(a)(iii), above. Thus, there was a known correlation between the function of the alphaviral replicases of the invention and their structure. The absence of multiple working examples cannot, by itself, support a *prima facie* case of insufficient written description.

(b) Satisfaction of the Written Description Requirement Does Not Require the Disclosure of All Mutations that Render Alphaviral Replicases Non-Cytopathic and Temperature Sensitive

The Examiner also stated that "the specification has failed to disclose what mutations are required to render any other RNA-dependent RNA polymerase both temperature sensitive and non-cytopathic, or what other mutations could confer this phenotype on the Sindbis virus polymerase." *See* Office Action dated June 17, 2003, page 4, lines 9-12. As mentioned above, it was known that mutations in the

nonstructural protein genes of alphaviruses produce non-cytopathicity and/or temperature sensitivity, and that a person of ordinary skill in the art, in view of the present specification, could have easily obtained nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases. Satisfaction of the written description requirement therefore does not require that all possible mutations that confer these phenotypes be recited in the specification.

The Examiner stated that:

> [t]he instant application does not provide a written description that would allow one of skill in the art to immediately *envisage* the specific structure for Sindbis virus non-cytopathic, temperature sensitive replicase, or for the broader genus of alphaviral non-cytopathic, temperature-sensitive replicase.

Office Action dated June 17, 2003, page 5, lines 15-18 (emphasis added). Along these same lines, the Examiner also asserted that:

> The disclosed mutations do not provide a sufficient correlation between structure and function to allow one to *envisage* other temperature sensitive mutations, and therefore they do not provide an adequate written description of the genus of temperature sensitive alphaviral replicases.

Office Action dated June 17, 2003, page 8, lines 4-7 (emphasis added). Appellants respectfully submit that the proper legal standard for written description does not require that one of ordinary skill in the art be able to "envisage," *i.e.*, "to picture in the mind" (American Heritage Dictionary), the nucleic acid sequence of every nucleic acid molecule that is encompassed by or included within the present claims. The Examiner has failed to point to any legal authority to support such a restrictive view of the written

description requirement when the claims include a structural as well as a functional definition of the invention. Cases such as *Amgen* and *Fiers v. Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993), which were cited by the Examiner, involve claims/counts that defined the invention by *functional* language only. These cases therefore do not support a rejection of the present claims for insufficient written description.

In fact, the USPTO's own guidelines clearly indicate that functional characteristics *alone* may satisfy the written description requirement if there is a known or disclosed correlation between structure and function. *See* MPEP § 2163. This position has been explicitly endorsed by the Federal Circuit in the context of inventions involving genetic material. *See Enzo*, 296 F.3d at 1324, 63 USPQ2d at 1613.

Here, Appellants have provided, not only a functional description of the nucleic acid molecules of the invention (encoding a non-cytopathic, temperature sensitive alphaviral replicase), but also structural characteristics of the nucleic acid molecules (non-cytopathicity and temperature sensitivity being conferred by one or more mutations in the genes encoding the nonstructural proteins of the replicase). The claims of Group II provide even more structural definition, specifying that non-cytopathicity is conferred by one or more mutations in the nsP2 gene of the replicase, and/or that temperature sensitivity is conferred by one or more mutations in the nsP4 gene of the replicase. As demonstrated above, it was well known in the art that non-cytopathicity and temperature sensitivity are caused by mutations in the genes encoding the nonstructural proteins of alphaviral replicases. *See* section VIII.A.2(a)(iii), above. Thus, there is a known correlation between (a) non-cytopathicity and temperature sensitivity of alphaviral

replicases on the one hand, and (b) mutations in the genes encoding the nonstructural proteins of the replicase on the other.

Thus, when analyzed under the appropriate legal standard as specified by the Federal Circuit, and the USPTO's guidelines, it must be concluded that the written description requirement is fully satisfied for the claims on appeal. The Examiner's requirement for a detailed structural definition of every member of a claimed genus is not the proper legal standard and cannot stand as the basis for a rejection under 35 U.S.C. § 112, first paragraph.

B. Enablement

1. Legal Standard for Enablement

In order to satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph, Appellants' specification must enable any person skilled in the art to make and use the claimed invention without undue experimentation. *See In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). *See also United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988). The factors to be considered when determining whether the necessary experimentation is "undue" include: (a) the breadth of the claims, (b) the nature of the invention, (c) the state of the prior art, (d) the level of one of ordinary skill, (e) the level of predictability in the art, (f) the amount of direction provided by the inventor, (g) the existence of working examples, and (h) the quantity of experimentation needed to make or use the invention based on the content of the disclosure. *See Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. Moreover, as long as the specification discloses at least one method for making and using the claimed invention, then the enablement requirement of 35 U.S.C. § 112, first

paragraph is satisfied. *See Johns Hopkins Univ. v. CellPro, Inc.*, 152 F.3d 1342, 1361, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998).

An Applicant is not limited to the confines of the specification to provide the necessary information to enable an invention. *See In re Howarth*, 654 F.2d 103, 105-6, 210 USPQ 689, 692 (CCPA 1981). An Applicant need not supply information that is well known in the art. *See Genentech, Inc. v. Novo Nordisk*, 108 F.3d 1361, 1366, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997); *Howarth*, 654 F.2d at 105-6, 210 USPQ at 692; *see also In re Brebner*, 455 F.2d 1402, 173 USPQ 169 (CCPA 1972) (finding a disclosure enabling because the procedure for making the starting material, although not disclosed, would have been known to one of ordinary skill in the art as evidenced by a Canadian patent). "'That which is common and well known is as if it were written out in the patent and delineated in the drawings.'" *Howarth*, 654 F.2d at 106, 210 USPQ at 692 (quoting *Webster Loom Co. v. Higgins* et al., 105 U.S. (15 Otto.) 580, 586 (1881)). Moreover, one of ordinary skill in the art is deemed to know not only what is considered well known in the art but also where to search for any needed starting materials. *See Id.*

In order to establish a *prima facie* case of lack of enablement, the Examiner has the initial burden to set forth a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). To satisfy this burden, "it is incumbent upon the Patent Office. . . to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See In re Marzocchi*, 439 F.2d 220, 224, 169

USPQ 367, 370 (CCPA 1971) (emphasis in original). As enunciated by the Federal Circuit:

> [A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

In re Brana, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (emphasis in original; quoting *Marzocchi*, 439 F.2d at 224, 169 USPQ at 370).

2. The Subject Matter of the Claims of Group I (Claims 75-78, 81-84, 86-101, 103, 105-107 and 109-136) is Fully Enabled

A person of ordinary skill in the art would have been able to make and use the full scope of the subject matter encompassed by the claims of Group I using only routine methods. More specifically, it would have required only routine experimentation to obtain nucleic acid molecules that encode, *inter alia*, a non-cytopathic, temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of the replicase. Thus, the enablement requirement of 35 U.S.C. § 112, first paragraph, is fully satisfied for the claims of Group I.

(a) Based on Examples in the Specification and The High Level of Sequence Homology Shared Among Alphaviral Nonstructural Proteins, A Person of Ordinary Skill in the Art Would Have Been Able to Make and Use the Full Scope of Subject Matter Encompassed by the Claims of Group I

The present specification describes in detail the construction of an exemplary nucleic acid molecule that encodes a non-cytopathic, temperature sensitive alphaviral replicase (pCYTts). *See* specification at page 41, line 2, through page 43, line 8 (Example 1). This exemplary nucleic acid molecule encodes a Sindbis virus replicase containing a Pro726Ser mutations in nsP2 and a Gly153Glu mutation in nsP4. In view of this working example, a person of ordinary skill in the art could have constructed, with only routine methods, additional nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases. For example, a skilled artisan, in view of the high level of sequence homology shared among nsPs from various alphaviruses (*see* section VIII.A.2(b), above), could have easily made mutations in the nsP genes of other alphaviruses which correspond to the nsP mutations described in the specification. The mutants could have been easily tested to confirm that they encoded non-cytopathic, temperature sensitive replicases. Such testing would not have been regarded as undue experimentation.

(b) A Person of Ordinary Skill in the Art Would Have Been Able to Make and Use the Full Scope of Subject Matter Encompassed by the Claims of Group I by Genetic Screening

(i) Genetic Screening Approaches for Producing Alphaviral Mutants with Particular Phenotypes Were Well Known in the Art as of the Effective Filing Date of the Application

In addition to using the working examples from the specification as a guide for creating nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases, a person of ordinary skill in the art could also have used genetic screening approaches.

Genetic screening approaches in general, and genetic screening approaches to produce temperature sensitive or non-cytopathic alphaviral mutants in particular, were well known to persons of ordinary skill in the art many years prior to the effective filing date of the present application. General methods for producing mutant proteins having particular desired phenotypes are described in Sambrook *et al.*, "Creating Many Mutations in a Defined Segment of DNA," in *Molecular Cloning, A Laboratory Manual*, Sambrook *et al.*, eds., Cold Spring Harbor Laboratory Press, pp. 15.95-15.108 (1989) (copy submitted with Appellants' Amendment and Reply Under 37 C.F.R. § 1.116, filed November 4, 2002, as Exhibit 1). Sambrook describes oligonucleotide-, chemical-, and polymerase-based mutagenesis methods. The methods of Sambrook could have been used to produce non-cytopathic, temperature sensitive alphaviral replicase mutants for use with the present invention.

In addition, a review of the scientific literature demonstrates that random mutagenesis and genetic screening approaches have been used for several years to

identify alphaviral mutants having specific desired phenotypes. *See, e.g.*, Keränen and Kääriäinen, *Acta Path. Microbiol. Scand. Sect. B, 82*:810-820 (1974) (copy submitted with the Reply Under 37 C.F.R. § 1.111, filed December 17, 2003, as Exhibit C). Keränen and Kääriäinen describe the identification of sixteen temperature sensitive Semliki Forest virus mutants. The mutants were identified by exposing virus particles to a mutagen and selecting for mutants that were defective in virus RNA synthesis at non-permissive temperatures.

Similarly, Weiss *et al., J. Virol. 33*:463-474 (1980) (copy submitted as document AR26 in the Information Disclosure Statement filed on June 28, 1999) used a genetic selection approach to identify Sindbis virus mutants capable of establishing persistent infection of BHK host cells (*i.e.*, non-cytopathic mutants). Subsequently, Dryga *et al.*, *Virology 228*:74-83 (1997) (copy submitted as document AS6 in the Information Disclosure Statement filed on June 28, 1999) identified the precise genetic change responsible for the non-cytopathic phenotype of the virus mutants identified by Weiss.

At the time of the effective filing date of the present application, a person of ordinary skill in the art, in view of the teachings of the present specification, could have combined the mutagenesis and selection approach used by Keränen and Kääriäinen (identifying temperature sensitive mutants) with that of Weiss (identifying non-cytopathic mutants) and Dryga to produce alphaviral replicases possessing both non-cytopathic and temperature sensitive phenotypes.

The Declaration of Dr. Schlesinger Under 37 C.F.R. § 1.132 (submitted with Appellants' Supplemental Reply, filed on March 31, 2003) provides additional support for Appellants' positions that (a) a person of ordinary skill in the art would have likely

used a mutagenesis and screening approach to obtain nucleic acid molecules encoding non-cytopathic, temperature-sensitive alphaviral replicases for use in the present invention, and (b) obtaining such nucleic acid molecules using a mutagenesis and screening approach would not have required undue experimentation.

As noted by the Federal Circuit, screening -- even screening that involves the generation of numerous negative outcomes -- is not deemed undue experimentation when those skilled in the art typically engage in such screening. *See Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. The references cited above and the Declaration of Professor Schlesinger show that persons of ordinary skill in the art typically engaged in genetic screening to identify alphavirus replicase mutants having desired phenotypes; *i.e.*, non-cytopathicity and temperature sensitivity. Under *Wands*, the production of nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases by genetic screening would not be regarded as involving undue experimentation.

(ii) Several Temperature Sensitive Alphaviral Replicases Were Known in the Art as of the Effective Filing Date of the Application

There are many examples in the art, prior to the effective filing date of the present application, of temperature sensitive alphaviral replicases that were created by mutagenesis and screening strategies. Examples include LaStarza *et al.*, *J. Virol. 68*:5781-5791 (1994) (describing the construction of random linker insertion mutations in Sindbis virus nsP3 to generate temperature sensitive mutants); Shirako and Strauss, *Virology 177*:54-64 (1990) (describing mutations created in the penultimate Gly in Sindbis virus nsP1 which resulted in temperature sensitivity); and Shirako and Strauss, *J. Virol. 72*:2310-2315 (1998) (describing N terminal mutations made in the nsP4 gene of

Sindbis virus which caused temperature sensitivity). The strategies used in these references would have been available to persons of ordinary skill in the art.

Moreover, in view of the guidance provided in the present specification, the temperature sensitive mutants that were known in the art (such as those described above) could have been combined with non-cytopathic mutations (*e.g.*, the Pro726Ser mutation disclosed in the specification, or other non-cytopathic mutations obtained through genetic screening approaches). The resulting combination of mutations would have resulted in nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases that could be used with the present invention. The ability of persons of ordinary skill in the art to combine temperature sensitive mutations with non-cytopathic mutations to produce mutant replicases having both phenotypes is described in section VIII.B.2(c)(ii), below.

(c) Expression Vectors Encoding Non-Cytopathic, Temperature Sensitive Alphaviral Replicases Have been Described In the Art After the Effective Filing Date of the Application, and Were Made Using Methods That Were Known and Available Prior to the Effective Filing Date of the Application

Publications dated after the effective filing date of an application can be used to support enablement when the later publications provide evidence of the state of the art existing on the filing date of the application. *See In re Hogan*, 559 F.2d 595, 605, 194 USPQ 527, 537 (CCPA 1977). As noted above, several non-cytopathic or temperature sensitive alphaviral replicase mutants were known in the art prior to the effective filing date of the application. Additional alphaviral expression vectors encoding non-cytopathic, temperature sensitive replicases have been described in publications dated after the effective filing date of the application. *See* section VIII.B.2(c)(ii), below. The

techniques, materials and information used to create the alphaviral expression vectors described in these post-filing date references, however, would have been available to persons of ordinary skill in the art *prior* to the effective filing date. Therefore, the examples in the art of the successful production of non-cytopathic, temperature sensitive alphaviral replicases demonstrate that making and using the nucleic acid molecules of the present invention would not have required undue experimentation.

(i) Several Non-Cytopathic Alphaviral Replicase Mutants Have Been Made Using Methods That Were Available to Persons of Ordinary Skill in the Art as of the Effective Filing Date of the Present Application

1) Weiss/Dryga

As discussed above, Weiss genetically selected for Sindbis viruses that persistently infected BHK cells. Briefly, Weiss infected BHK cells with a preparation of Sindbis virus containing "defective interfering" (DI) particles. Four days after infection, most of the infected cells had died. However, a small percentage of viable infected cells were recovered. From the viable cells, a non-cytopathic virus was isolated, designated SIN-1. In a subsequent publication, Dryga described the cDNA cloning and sequencing of the nonstructural protein genes of the SIN-1 virus. Dryga thereby identified the mutation in the Sinbis genome responsible for non-cytopathicity.

Both Weiss and Dryga were published prior to the effective filing date of the present application (March 27, 1998). Weiss was published in 1980, and Dryga was published in 1997. Therefore, the methods used by Weiss and Dryga would have been available to a person of ordinary skill in the art as of the effective filing date of the application. Using the teachings of the present specification, a person of ordinary skill in

the art could have used the screening and cloning methods described by Weiss and Dryga to obtain additional non-cytopathic alphavirus replicase mutations.

2) Agapov

An alternative method for identifying non-cytopathic alphaviral replicase mutants is described in Agapov *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 95*:12989-12994 (October, 1998) (cited by the Examiner on Form PTO-892 in the Office Action Dated August 15, 2000). Agapov transduced cells with Sindbis replicons containing the puromycin *N*-acetyltransferase (*PAC*) gene as a dominant selectable marker. Replicons were selected that produced populations of cells that were not cytopathic to cells (*i.e.*, replicons that produced pur^R cells). Using this selection method, a mutation was identified in the nsP2 gene in which Pro726 was changed to a Leu.

The methods used by Agapov would have been available to persons of ordinary skill in the art prior to the effective filing date of the present application. Methods for producing alphaviral replicons expressing selectable markers were known in the art. *See, e.g.*, Xiong *et al.*, *Science 243*:1188-1191 (1989) (describing Sindbis virus vectors engineered to express the chloramphenicol acetyltransferase (CAT) selectable marker gene) (copy submitted as document AS27 in the Information Disclosure Statement filed on June 28, 1999). Transfecting cells with a replicon expressing a selectable marker and selecting for mutants that are non-lethal to cells would have therefore been routine in the art. It is worth noting that Agapov was published only seven months after the effective filing date of the present application (March 27, 1998). The fact that Agapov was published soon after the effective filing date of the present application provides further

indication that the methods of Agapov were known and available prior to the effective filing date of the application.

Persons of ordinary skill in the art, in view of the teachings of the present specification, could have used the genetic selection approach of Agapov to obtain additional non-cytopathic alphaviral replicase mutations.

3) Perri

Perri *et al., J. Virol. 74*:9802-9807 (2000) (copy submitted with Appellants' Supplemental Reply filed on March 31, 2003, as Exhibit 12) describes another method for obtaining non-cytopathic alphaviral replicases using methods that would have been available to persons of ordinary skill in the art prior to the effective filing date of the present application. Perri used a genetic screening approach that resulted in the identification of five non-cytopathic alphaviral replicase mutants. To identify the mutants, Perri placed the neomycin phospotransferase gene (*neo*) under the control of the subgenomic promoter in both Sindbis virus- and Semliki Forest virus-derived replicons. Some of the DNA templates were subjected to random mutagenesis. Replicons were transcribed from the DNA templates, and the RNA replicons were transfected into BHK cells. The transfected cells were subjected to G418 selection; persistent, drug resistant colonies were selected in order to identify non-cytopathic replicons.

Using this method, Perri identified five non-cytopathic mutations, two in the Sindbis virus nsP2 gene, and three in the Semliki Forest virus nsP2 gene. For Sindbis virus replicons, the mutations were A1E ("S1") and P726T ("S2"). For Semliki Forest virus replicons, the mutations were L10T ("SF2A"), a deletion of D469 ("SF1B") and

L713P ("SF2C"). *See* Perri at 9804, Figure 2C. Thus, using a genetic screening approach, Perri was able to easily identify five non-cytopathic alphaviral replicases.

The methods used by Perri would have been available to persons of ordinary skill in the art prior to the effective filing date of the present application. As mentioned above, methods for producing alphaviral replicons expressing selectable markers were known in the art. Procedures for mutagenizing nucleic acid molecules were also known in the art. *See, e.g.*, Sambrook (discussed in section VIII.B.2(b)(i), above). Transfecting host cells with the mutagenized replicons and selecting for drug-resistant colonies would have involved only routine methods. Therefore, the methods of Perri could have been used by persons of ordinary skill in the art, prior to the effective filing date of the present application, to obtain additional non-cytopathic alphaviral replicase mutations.

(ii) Non-Cytopathic, Temperature Sensitive Alphaviral Replicase Mutants Have Been Created by Combining Known Replicase Mutants, Using Methods That Were Available to Persons of Ordinary Skill in the Art as of the Effective Filing Date of the Present Application

There are two examples from the scientific literature of the production of alphaviral vectors that express non-cytopathic, temperature sensitive alphaviral replicases. In both examples, the nucleic acid molecules encoding the non-cytopathic, temperature sensitive alphaviral replicases were produced by combining known alphaviral replicase mutations. Standard molecular biological techniques (available in the art prior to the effective filing date of the present application) were used to create the combination mutants. Thus, these two examples demonstrate that, with the guidance of the present specification, the production of nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases could have been accomplished

prior to the effective filing date of the present application without undue experimentation.

The first example is Lundstrom *et al.*, *Histochem. Cell. Biol. 115*:83-91 (2001) ("Lundstrom 2001") (copy submitted with Appellants' Supplemental Reply filed on December 17, 2003, as Exhibit A). Lundstrom 2001 describes the combination of a known non-cytopathic mutation, referred to as SFV(PD) (which has two point mutations in the nsP2 gene), with previously known temperature sensitive mutations. As expected, the combination mutants exhibited both non-cytopathic and temperature sensitive phenotypes.

The second example is Lundstrom *et al.*, *Mol. Ther. 7*:202-209 (2003) ("Lundstrom 2003") (copy submitted with Appellants' Supplemental Reply filed on December 17, 2003, as Exhibit B). Lundstrom 2003 describes the combination of the SFV(PD) mutation with Perri's L713P nsP2 non-cytopathic mutation. The resulting combination mutant, referred to as SFV(PD713P), exhibited both non-cytopathic and temperature sensitive phenotypes.

Both Lundstrom 2001 and Lundstrom 2003 used PCR-based site-directed mutagenesis techniques to produce the combination mutants. PCR-based site directed mutagenesis techniques were well known in the art prior to the effective filing date of the present application. *See*, *e.g.*, Stappert, J., "Methods for Generating Multiple Site-Directed Mutations *In Vitro*," in *PCR Technology: Current Innovations*, Griffin and Griffin, eds., CRC Press, pp. 59-67 (1994) (copy submitted herewith as Exhibit 1). The methods described in the Lundstrom references would have been available to persons of ordinary skill in the art, prior to the effective filing date of the present application, and

would have enabled the production of nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases. The Lundstrom references demonstrate that alphaviral replicase mutants exhibiting both temperature sensitivity and non-cytopathicity could have been easily made by combining previously known mutations in nsP genes.

(d) Summary

The above discussion illustrates the following points in support of the enablement of the present invention:

1. Several temperature sensitive alphaviral replicase mutations were known in the art prior to the effective filing date of the present invention;
2. Several non-cytopathic alphaviral replicase mutant have been described in the art. The methods used to obtain the non-cytopathic replicase mutations would have been available to persons of ordinary skill in the art prior to the effective filing date of the present invention; and
3. Alphaviral expression vectors encoding non-cytopathic, temperature sensitive alphaviral replicases have been obtained by combining, *e.g.*, known temperature sensitive mutations with known non-cytopathic mutations. The methods used to combine the known mutations would have been available to persons of ordinary skill in the art prior to the effective filing date of the present invention.

Therefore, at the time of the effective filing date of the present invention, a person of ordinary skill in the art could have produced alphaviral expression vectors encoding non-cytopathic, temperature sensitive alphaviral replicases using routine

methods. Such methods would not have involved undue experimentation. Accordingly, the subject matter of the claims of Group I is fully enabled.

3. *The Subject Matter of the Claims of Group II (Claims 137-145) is Fully Enabled*

The claims of Group II depend from Group I claims 75, 103 and 125. The subject matter of the claims of Group II is fully enabled for at least the same reasons that the subject matter of the claims of Group I is enabled. *See* discussion in section VIII.B.2, above. In addition, the claims of Group II specify that non-cytopathicity is conferred by one or more mutations in the nsP2 gene of the replicase, and/or that temperature sensitivity is conferred by one or more mutations in the nsP4 gene of the replicase. Thus, in order to obtain nucleic acid molecules for use with the invention defined by the claims of Group II, a person of ordinary skill in the art would have been directed to the specific genes of the alphaviral replicase in which to concentrate his or her mutagenesis efforts. The subject matter of the claims of Group II is therefore fully enabled. (Appellants note that the claims of Group II were not rejected for lack of enablement.)

4. *The Examiner Has Not Established a Prima Facie Case of Non-Enablement*

(a) *A Person of Ordinary Skill in the Art Would Not have Been Required to Predict the Effects of Mutations on Protein Function in Order to Make Nucleic Acid Molecules that Encode Non-Cytopathic, Temperature Sensitive Alphaviral Replicases*

The Examiner has not established a *prima facie* case of non-enablement. The Enablement rejection is based on the Examiner's assertion that it is difficult to predict the relationship between nucleic acid mutations and protein function. *See* Office Action

dated June 17, 2003, page 11, lines 14-19. Apparently, the Examiner believes that, in order to produce additional nucleic acid molecules encoding non-cytopathic, temperature-sensitive alphaviral replicases, a skilled artisan would have needed to make individual site-directed mutations in the replicase genes, and would have needed to know *a priori* that the mutations caused the desired phenotypes. Appellants respectfully disagree.

As discussed in section VIII.B.2(c), above, alphaviral vectors encoding non-cytopathic, temperature sensitive replicases -- in addition to the exemplary embodiment described in the present specification -- have been described in the art. The methods that were used to produce these vectors would have been known and available to persons of ordinary skill in the art prior to the effective filing date of the present application. The methods that were used to produce these vectors did not involve predicting the effects of particular mutations on protein function. Rather, as with the exemplary embodiment in the present specification, the non-cytopathic, temperature sensitive vectors that were described in the art after the effective filing date of the application were produced by combining known mutations that individually caused non-cytopathicity and/or temperature sensitivity. The individual non-cytopathic and temperature sensitive mutations were produced using classical genetic screening approaches that were known and available prior to the effective filing date of the present application. The mutations were combined using well known molecular biological techniques that were also known and available prior to the effective filing date of the present application.

Making and using the non-cytopathic, temperature sensitive alphaviral vector exemplified in the present application (pCYTts) and the vectors described by others after

the effective filing date of the present application did not require the ability to predict protein function from protein structure. Thus, the Examiner's comments regarding the ability of persons of ordinary skill to predict "which amino acid substitutions will confer temperature sensitivity and non-cytopathicity on a given polymerase" cannot support a *prima facie* case of non-enablement.

(b) The Results Obtained By Combining Mutations in a Yeast F1-ATPase Cannot be Used to Draw Conclusions About the Predictability of Combining Mutations in Alphaviral Replicases

To further support the enablement rejection, the Examiner has cited the results that were observed when multiple mutations in the yeast F1-ATPase beta subunit were combined with one another. *See* Office Action dated June 17, 2003, pages 11-12. According to the Examiner, combining mutations in the yeast F1-ATPase beta subunit "produced totally unpredictable results." Appellants respectfully submit that the results observed by combining mutations in a yeast F1-ATPase do not provide any indication as to the effects of combining mutations in alphaviral replicases. Although the experiments cited by the Examiner, relating to a yeast F1-ATPase, produced unpredictable results, the examples described in the present application, and the examples provided by Lundstrom 2001 and Lundstrom 2003 produced entirely predictable results. That is, the combination of alphaviral replicase mutations produced alphaviral replicases that had both temperature sensitive and non-cytopathic phenotypes.

In summary, the present claims relate to alphaviral expression vectors encoding non-cytopathic, temperature sensitive alphaviral replicases. The combination of non-cytopathic mutations with temperature sensitive mutations, as described by the present inventors and by others, resulted in nucleic acid molecules encoding non-cytopathic,

temperature sensitive alphaviral replicases, as expected. The present claims do not relate to yeast F1-ATPases. Thus the results cited by the Examiner for a yeast F1-ATPase cannot support a *prima facie* case of non-enablement.

(c) The Sindbis Virus Replicase Mutations Described in the Specification Would Have Enabled the Production of Non-Cytopathic, Temperature Sensitive Replicases in Other Alphaviruses Due to the High Degree of Sequence Homology that is Shared Among Nonstructural Proteins of Alphaviruses

To Support the enablement rejection, the Examiner further stated that:

> Applicant has disclosed mutations only of a Sindbis virus replicase, whereas the claims encompass replicases from all alphaviruses. One of skill in the art could not predict which, if any, of these replicases could be mutated to be appropriately temperature sensitive and non-cytopathic, or what mutations would be required for this.

Office Action dated June 17, page 12, line 21, through page 13, line 2. Appellants again note that a person of ordinary skill in the art could have obtained nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases using genetic screening approaches. Such approaches could have been applied to any alphaviral replicase, regardless of the virus from which it was derived. In addition, alphavirus replicases share a great deal of sequence homology with one another. *See* Exhibits 13-18, submitted with Appellants' Supplemental Reply filed on March 31, 2003. Mutations in one alphaviral species which cause non-cytopathicity and/or temperature sensitivity would guide the skilled artisan to similar mutations at homologous genetic loci in other species to produce these phenotypes. Thus, the fact that the claims are not limited to non-cytopathic, temperature sensitive replicases from any particular alphavirus does not support a finding of non-enablement.

(d) Several Mutations Have been Identified that Render Alphaviral Replicases Non-Cytopathic, And Such Mutations Have Been Combined with Other Replicase Mutations to Produce Non-Cytopathic, Temperature Sensitive Alphaviral Replicases

Finally, the Examiner stated that "only a single alphaviral replicase mutation conferring non-cytopathicity has been identified, nsp2 P726S." *See* Office Action dated June 17, 2003, page 11, lines 5-6. Applicants respectfully point out that this is an incorrect statement. As discussed above, multiple non-cytopathic alphaviral replicase mutants have been identified by Weiss, Dryga, Agapov and Perri. *See* section VIII.B.2(c)(i), above. Non-cytopathic mutations of Perri and Lunstrom 1999 have been combined with other replicase mutations to produce nucleic acid molecules that encode non-cytopathic, temperature sensitive alphaviral replicases. *See* section VIII.B.2(c)(ii), above. Thus, the enablement rejection, insofar as it depends on the Examiner's position that only one non-cytopathic alphaviral replicase mutant has been identified, cannot be maintained.

(e) Summary

The Examiner has not presented sufficient evidence or scientific reasoning to demonstrate that it would have required undue experimentation to produce additional nucleic acid molecules encoding non-cytopathic, temperature sensitive alphaviral replicases. The fact that others in the art were able to produce additional non-cytopathic, temperature sensitive alphaviral replicases, using general methods that would have been available prior to the effective filing date of the present application, indicates that the production of such replicases would not have been regarded as involving undue experimentation. Thus, a *prima facie* case of non-enablement has not been established.

IX. Conclusion

In view of the foregoing remarks, Appellants respectfully request that the Board reverse the Examiner's 35 U.S.C. 112, first paragraph, rejections of claims 75-78, 81-84, 86-101, 103, 105-107 and 109-145 and remand this application for issue.

Respectfully submitted,
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X. *Appendix (37 C.F.R. § 1.192(c)(9))*

75. A DNA molecule which encodes an RNA molecule comprising:

(a) at least one *cis*-acting sequence element,

(b) a first open reading frame which encodes a non-cytopathic temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of said replicase, and

(c) at least one second nucleotide sequence selected from the group consisting of:

(i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a translatable format after one or more RNA-dependent RNA replication events;

(ii) a sequence complementary to all or part of the second open reading frame of (i); and

(iii) a sequence encoding an untranslated RNA molecule, or complement thereof;

wherein said second nucleotide sequence is operably linked to a promoter which is recognized by said non-cytopathic, temperature-sensitive alphaviral replicase.

76. The DNA molecule of claim 75, which comprises one second nucleotide sequence.

77. The DNA molecule of claim 75, wherein said second open reading frame is in a translatable format after one RNA-dependent RNA replication event.

78. The DNA molecule of claim 75, wherein said second open reading frame is in a translatable format after three RNA-dependent RNA replication events.

81. The DNA molecule of claim 75, wherein said alphaviral replicase is derived from a Sindbis virus.

82. The DNA molecule of claim 75 which encodes an alphaviral replicase having replicase activity at 34°C which is at least five fold lower than the replicase activity exhibited at 29°C.

83. The DNA molecule of claim 75, wherein the second open reading frame encodes a cytokine, a lymphokine, a tumor necrosis factor, an interferon, a toxic protein, or a prodrug converting enzyme.

84. The DNA molecule of claim 75, wherein the second open reading frame encodes human erythropoietin or human β-interferon.

86. A method of making a recombinant host cell comprising introducing the DNA molecule of claim 75 into a host cell *in vitro*.

87. An *in vitro* cell culture comprising a recombinant host cell produced by the method of claim 86.

88. An *in vitro* cell culture comprising a recombinant host cell comprising the DNA molecule of claim 75.

89. The cell culture of claim 88, wherein some or all of the DNA molecule is stably maintained in said host cell.

90. An RNA molecule transcribed from the DNA molecule of claim 75.

91. An alphaviral particle containing the RNA molecule of claim 90.

92. An *in vitro* cell culture comprising a recombinant host cell comprising the RNA molecule of claim 90.

93. A method for producing a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) introducing at least one DNA molecule of claim 75 into said host cells *in vitro*;

(b) culturing said host cells under conditions suitable for expression of said protein or untranslated RNA molecule; and

(c) recovering said protein or untranslated RNA molecule;

wherein said protein or untranslated RNA molecule is encoded by said DNA molecule.

94. A method for producing a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) introducing at least one RNA molecule of claim 90 into said host cells in vitro;

(b) culturing said host cells under conditions suitable for expression of said protein or untranslated RNA molecule; and

(c) recovering said protein or untranslated RNA molecule;

wherein said protein or untranslated RNA molecule is encoded by said RNA molecule.

95. The method of claim 94, wherein the protein is erythropoietin.

96. The method of claim 94, wherein said RNA is packaged into an alphaviral particle.

97. A method for producing alphaviral particles, said method comprising:

(a) introducing into a host cell *in vitro* at least one DNA molecule of claim 75 having one or more open reading frames which encode alphaviral structural proteins;

(b) growing host cells under culture conditions suitable for the production of alphaviral particles which contain an RNA transcription product of said DNA molecule; and

(c) recovering said alphaviral particles.

98. A method for producing a protein encoded by RNA contained in an alphaviral particle produced by the method of claim 97 in a recombinant host cell comprising:

(a) infecting a host cell *in vitro* with the alphaviral particle;

(b) growing said host cell under culture conditions suitable for the production of said protein; and

(c) recovering said protein.

99. The method of claim 98, wherein said protein is erythropoietin.

100. A method for regulating the expression of a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one DNA molecule of claim 75 into said host cells *in vitro*; and

(c) changing the temperature of the host cell culture from:

(i) a permissive temperature to a restrictive temperature, or

(ii) a restrictive temperature to a permissive temperature;

wherein said protein or untranslated RNA molecule is encoded by said DNA molecule.

101. A method for regulating the expression of a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one RNA molecule of claim 90 into said host cells *in vitro*; and

(c) changing the temperature of the host cell culture from:

(i) a permissive temperature to a restrictive temperature, or

(ii) a restrictive temperature to a permissive temperature;

wherein said protein or untranslated RNA molecule is encoded by said RNA molecule.

103. A DNA vector system comprising one or more polynucleotides which encode RNA molecules, said RNA molecules comprising:

(a) at least one *cis*-acting sequence element,

(b) a first open reading frame having a nucleotide sequence encoding a non-cytopathic, temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of said replicase, and

(c) at least one second nucleotide sequence selected from the group consisting of:

(i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a translatable format after one or more RNA-dependent RNA replication events;

(ii) a sequence complementary to all or part of the second open reading frame of (i); and

(iii) a sequence encoding an untranslated RNA molecule, or complement thereof;

wherein said second nucleotide sequence is operably linked to a promoter which is recognized by said non-cytopathic, temperature-sensitive alphaviral replicase.

105. The DNA vector system of claim 103 which encodes an alphaviral replicase having replicase activity at 34°C which is at least five fold lower than the replicase activity exhibited at 29°C.

106. The DNA vector system of claim 103, wherein the second open reading frame encodes a cytokine, a lymphokine, a tumor necrosis factor, an interferon, a toxic protein, or a prodrug converting enzyme.

107. The DNA vector system of claim 103, wherein the second open reading frame encodes human erythropoietin or human β-interferon.

109. A method of making a recombinant host cell comprising introducing at least one polynucleotide of claim 103 into a host cell *in vitro*.

110. An *in vitro* cell culture comprising a recombinant host cell produced by the method of claim 109.

111. An *in vitro* cell culture comprising a recombinant host cell comprising at least one polynucleotide of claim 103.

112. The cell culture of claim 111, wherein some or all of the polynucleotide sequences of claim 103 are stably maintained in said host cell.

113. A composition comprising one or more RNA molecules transcribed from one or more polynucleotides of the vector system of claim 103.

114. An alphaviral particle containing at least one RNA molecule of claim 113.

115. An *in vitro* cell culture comprising a recombinant host cell comprising at least one RNA molecule of claim 113.

116. A method for producing a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one DNA molecule of claim 103 into said host cells *in vitro*;

(c) recovering said protein or untranslated RNA molecule;

wherein said protein or untranslated RNA molecule is encoded by said DNA molecule.

117. A method for producing a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one RNA molecule of claim 113 into said host cells *in vitro*; and

(c) recovering said protein or untranslated RNA molecule;

wherein said protein or untranslated RNA molecule is encoded by said RNA molecule.

118. The method of claim 117, wherein the protein is erythropoietin.

119. The method of claim 117, wherein said RNA is packaged into an alphaviral particle.

120. A method for producing an alphaviral particle comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing into said host cells *in vitro* at least one DNA molecule of claim 103 having one or more open reading frames which encode alphaviral structural proteins;

(c) producing an alphaviral particle; and

(d) recovering said alphaviral particle.

121. A method for producing a protein in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) infecting said host cells *in vitro* with an alphaviral particle produced by the method of claim 120; and

(c) recovering said protein;

wherein said protein is encoded by nucleic acid contained in said alphaviral particle.

122. The method of claim 121, wherein said protein is erythropoietin.

123. A method for regulating the expression of a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one DNA molecule of claim 103 into said host cells *in vitro*; and

(c) changing the temperature of the host cell culture from:

(i) a permissive temperature to a restrictive temperature, or

(ii) a restrictive temperature to a permissive temperature;

wherein said protein or untranslated RNA molecule is encoded by said DNA molecule.

124. A method for regulating the expression of a protein or an untranslated RNA molecule in a recombinant host cell comprising:

(a) growing host cells under suitable culture conditions;

(b) introducing at least one RNA molecule of claim 111 into said host cells *in vitro*; and

(c) changing the temperature of the host cell culture from:

(i) a permissive temperature to a restrictive temperature, or

(ii) a restrictive temperature to a permissive temperature;

wherein said protein or untranslated RNA molecule is encoded by said RNA molecule.

125. A composition comprising one or more RNA molecules, said RNA molecules comprising:

(a) at least one *cis*-acting sequence element,

(b) a first open reading frame having a nucleotide sequence encoding a non-cytopathic, temperature-sensitive alphaviral replicase, wherein non-cytopathicity and temperature sensitivity are conferred by one or more mutations in the genes encoding the nonstructural proteins of said replicase, and

(c) at least one second nucleotide sequence selected from the group consisting of:

(i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a

translatable format after one or more RNA-dependent RNA replication events;

(ii) a sequence complementary to all or part of the second open reading frame of (i); and

(iii) a sequence encoding an untranslated RNA molecule, or complement thereof;

wherein said second nucleotide sequence is operably linked to a promoter which is activated by said non-cytopathic, temperature-sensitive alphaviral replicase.

126. The DNA molecule of claim 75, wherein said alphaviral replicase is derived from a Semliki Forest Virus.

127. The DNA molecule of claim 75, wherein said alphaviral replicase is derived from an Aura virus.

128. The DNA molecule of claim 75, wherein said alphaviral replicase is derived from a virus selected from the group consisting of Bebaru virus, Cabassou virus, Chikungunya virus, Eastern equine encephalomyelitis virus, Fort Morgan virus, Getah virus, Kyzylagach virus, Mayoaro virus, Middleburg virus, Mucambo virus, Ndumu virus, Pixuna virus, Tonate virus, Triniti virus, Una virus, Western equine encephalomyelitis virus, Whataroa virus, Venezuelan equine encephalomyelitis virus (VEE), and Ross River virus.

129. The DNA vector system of claim 103, wherein said alphaviral replicase is derived from a Sindbis virus.

130. The DNA vector system of claim 103, said alphaviral replicase is derived from a Semliki Forest Virus.

131. The DNA vector system of claim 103, said alphaviral replicase is derived from an Aura virus.

132. The DNA vector system of claim 103, wherein said alphaviral replicase is derived from a virus selected from the group consisting of Bebaru virus, Cabassou virus, Chikungunya virus, Eastern equine encephalomyelitis virus, Fort Morgan virus, Getah virus, Kyzylagach virus, Mayoaro virus, Middleburg virus, Mucambo virus, Ndumu virus, Pixuna virus, Tonate virus, Triniti virus, Una virus, Western equine encephalomyelitis virus, Whataroa virus, Venezuelan equine encephalomyelitis virus (VEE), and Ross River virus.

133. The RNA molecule of claim 125, wherein said alphaviral replicase is derived from a Sindbis virus.

134. The RNA molecule of claim 125, wherein said alphaviral replicase is derived from a Semliki Forest Virus.

135. The RNA molecule of claim 125, wherein said alphaviral replicase is derived from an Aura virus.

136. The RNA molecule of claim 125, wherein said alphaviral replicase is derived from a virus selected from the group consisting of Bebaru virus, Cabassou virus, Chikungunya virus, Eastern equine encephalomyelitis virus, Fort Morgan virus, Getah virus, Kyzylagach virus, Mayoaro virus, Middleburg virus, Mucambo virus, Ndumu

virus, Pixuna virus, Tonate virus, Triniti virus, Una virus, Western equine encephalomyelitis virus, Whataroa virus, Venezuelan equine encephalomyelitis virus (VEE), and Ross River virus.

137. The DNA molecule of claim 75, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase.

138. The DNA molecule of claim 75, wherein temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

139. The DNA molecule of claim 75, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase, and temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

140. The DNA vector system of claim 103, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase.

141. The DNA vector system of claim 103, wherein temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

142. The DNA vector system of claim 103, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase, and temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

143. The composition of claim 125, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase.

144. The composition of claim 125, wherein temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

145. The composition of claim 125, wherein non-cytopathicity is conferred by one or more mutations in the nsP2 gene of said replicase, and temperature sensitivity is conferred by one or more mutations in the nsP4 gene of said replicase.

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